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Covalent Binding and other Mechanisms of Primaquine Toxicity

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Abstract

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A dose-dependent covalent binding of primaquine to mouse liver microsomal protein was found in both *in vitro* and *in vivo* studies. The binding *in vitro* was linear during the first 90 minutes of incubation and dependent on the concentration of mouse liver microsomal protein. The *in vitro* covalent binding was markedly enhanced by co-factors. Pretreatment of mice with phenobarbital, a cytochrome P450 inducer, increased the microsomal cytochrome P450 content and the *in vitro* covalent binding significantly. Binding was markedly inhibited when microsomal metabolism inhibitors (SK&F-525A, metyrapone and piperonyl butoxide), and sulfhydryl-containing compounds were added to the incubation system. Conversely, the inhibitory effect of inhibitors was not shown on *in vivo* binding; also induction with phenobarbital decreased the extent of covalent binding *in vivo*. These findings pointed to the possibility that the extent of *in vivo* formation of metabolites which can bind covalently may be limited. However, injection of PQ (80 mg/Kg) into mice decreased the glutathione content of liver and erythrocytes, possibly through the formation of reactive metabolites.

The NADPH-induced peroxidation of lipid of mouse microsomes and human erythrocyte membranes were markedly inhibited by PQ and 5H6DPQ (a PQ derivative), respectively. In contrast, PQ and its derivatives enhanced the NADPH-induced peroxidation of the lipids of intact erythrocytes.

A significant amount of PQ metabolite(s) (formed by the mouse liver microsomal cofactor system) was bound covalently to the total protein of both normal and G6PD-deficient erythrocytes. The net binding to the protein of G6PD-deficient red cells was significantly greater than that to normal cells. A measurable amount of covalent binding of PQ metabolite(s) to human hemoglobin was also demonstrated. A small but significant amount of PQ metabolite(s) was also bound to human erythrocyte membrane protein. PQ and its derivatives decreased the amount of measurable sulfhydryl groups of G6PD-deficient human erythrocyte membranes significantly but had no effect on the -SH groups of normal cell membranes. These results suggest that covalent binding of PQ metabolite(s) to human erythrocyte total protein and membrane protein as well as to hemoglobin may play an important role in hemolysis, particularly after cellular GSH and membrane sulfhydryl groups are depleted.

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Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

Page

CHAPTER 1: GENERAL INTRODUCTION

Glucose-6-Phosphate Dehydrogenase Deficiency

The occurrence of a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) in human erythrocytes was discovered during investigations in Chicago (Carson et al., 1956) of acute hemolytic anemia which occurred in about 10% of American Negroes after administration of the 6-methoxy-8-aminoquinoline antimalarial drug, primaquine (PQ). This was 30 years after the first reported case of hemolysis induced by the related drug, pamaquine.

G6PD is the first enzyme in the pentose phosphate pathway (PPP). It catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone by NADP, which is reduced to NADPH. This is the main regulatory step of the PPP. Approximately 10% of glucose-6-phosphate is metabolized by the PPP in normal, unstressed erythrocytes, while the other 90% is metabolized by the Embden-Meyerhof pathway of anaerobic glycolysis (Murphy, 1960).

G6PD-deficient erythrocytes have a diminished G6PD activity and other related metabolic abnormalities (Tarlov, et al., 1962; Carson and Frischer, 1966; Beutier, 1978): (1) Abnormalities of the pentose phosphate pathway: pentose phosphate metabolism is deficient; NADPH regeneration capacity is impaired; reduced glutatione (GSH) content is reduced and GSH is more vulnerable to oxidation; glutathione reductase activity is increased due to a compensatory mechanism or a younger erythrocyte population; O_2 consumption is reduced and rate of

glucose utilization is diminished; lipid content is decreased; rate of methemoglobin reduction is diminished; methemoglobinemia is increased and cells are more susceptible to Heinz body formation; (2) Abnormalities of Embden-Meyerhof Pathway: increased aldolase activity; decreased NADPH/NADP ratio and fall in ATP content in vitro; (3) Diminished catalase activity.

Besides the biochemical abnormalities in G6PD-deficient erythrocytes, Afolayan (1979) found that the reactivity of lipids and proteins of normal red cell (G6PD A) membranes, preincubated with PQ or chloroquine with trinitrobenzene sulphonate, is less than that of G6PD-deficient red cell (G6PD A-) membranes. The fluorescence studies in the presence of PQ or chloroquine also suggested a possible structure difference between the two types of ghosts.

The severity of hemolysis that occurs after ingestion of certain hemolytic drugs, chemicals, or plants is influenced by the concentration of drug in the blood; viral and bacterial infections; diabetic acidosis; hypoglycemia of the newborn; age of the erythrocytes and schedule of drug administration (Tarlov et al., 1962).

The deficiency in G6PD activity may be caused by decreased synthesis of enzyme molecules, reduced stability of enzyme molecules, decreased catalytic activity, the absence of activators or coenzymes essential for G6PD activity, the presence of inhibitors, or a combination of two or more of these mechanisms (Tarlov et al., 1962; Carson and Frischer, 1966; Beutler, 1969, 1982).

Until 1982, about 147 variants of G6PD have been characterized

(Beutler, 1982) according to the criteria set forth by the World Health Organization scientific group: (1) red cell G6PD activity; (2) electrophoretic mobility in different buffers; (3) the Michaelis constants for its substrates, glucose-6-phosphate and NADP; (4) capacity to utilize 2-deoxy glucose-6-phosphate, galactose-6-phosphate, and deamino NADP and NAD; (5) heat lability; and (6) pH optimum.

G6PD B is the normal enzyme and is the most common type with a half life of 63 days. The most common types of G6PD deficient variants found in human erythrocytes are G6PD A, with 84% of normal enzyme activity; A-, with 5 to 15% of normal enzyme activity; Canton, with 0 to 5% of normal enzyme activity; and Mediterranean, with almost no enzyme activity in mature erythrocytes (Haut, 1980).

G6PD deficiency in the Caucasian and Oriental is more severe than that in the Negro. Hemolysis can be produced by very mild oxidant stress in patients with the Mediterranean type variant. A small number of G6PD-deficient subjects have a very rare variant. They show the clinical picture of nonspherocytic congenital hemolytic anemia, but they experience hemolytic anemia in the absence of exposure to any stress (Beutler, 1969; Tarlov et al., 1962).

G6PD-deficiency has a world-wide distribution. The incidence is highest in tropical and semi-tropical areas. The geographic distribution of this most common red cell enzyme deficiency tends to parallel the occurence of Plasmodium falciparium malaria. Moreover, G6PD-deficient erythrocytes are less frequently parasitized than

normal red cells *in vitro* (Luzzatto *et al.*, 1969). The frequency of the deficient gene is approximately 10% among American Negroes (Hockwald *et al.*, 1952), 5.5% among Southern Chinese (Chan *et al.*, 1964) and a high incidence among Southern Mediterranean people (Szeinberg *et al.*, 1958). Seldom are cases reported from Northern Europe.

The geographic distribution of G6PD-deficiency suggests an selective evolutionary advantage against malaria (Allison and Clyde, 1961). GSH (Trager, 1941; Friedman, 1979) and the PPP (Trager, 1941) are essential for optimum growth of malaria parasites. The low GSH content and the diminished PPP activity of G6PD-deficient erythrocytes are not adequate for survival of the invading parasites. When sera, collected from normal subjects at certain times after ingestion of a meal of fava beans, were added to cultures of *P. falciparum* in normal, G6PD-deficient, or β-thalassemia trait red cells, Friedman (1983) discovered that some sera were inhibitory to parasites in variant cells but not to those in normal red cells.

The recent study by Luzzatto *et al.* (1983) indicated that Plasmodium falciparum invades G6PD-deficient erythrocytes (Mediterranean variant) normally in culture but maturation of intracellular parasites is delayed and impaired, especially if the red cells are under oxidative stress. An adaptive change on the part of the parasites has been found since the invading parasites have a normal behaviour in the next cycle in G6PD-deficient erythrocytes.

Childs *et al.* (1958) reported that the structural gene for

G6PD-deficiency is sex-linked. The gene is located on the X-chromosome close to the locus for color blindness (Adam, 1961; Siniscalco and Filippi, 1964). The gene can be fully expressed in hemizygous male subjects ($\bar{X}Y$) and rarely in homozygous females ($\bar{X}\bar{X}$). Heterozygous female subjects ($\bar{X}X$) have one cell population with normal enzyme activity and another population with deficient activity (Beutler, 1982). Therefore, the phenotype activity is either normal, intermediate, or low as in homozygotes or hemizygotes (Tarlov et al., 1962). Only one of the two X chromosomes present in each somatic cell of the female is active, the other one being inactivated (Lyon hypothesis).

Most G6PD-deficient individuals have no overt symptoms under normal conditions since there is enough G6PD activity to protect the cells in the normal physiologic state. However, when these subjects are under oxidant stress (exposed to certain drugs, chemicals or plants), this deficiency increases the susceptibility of the red cells to destruction.

Primaquine Metabolism

Primaquine (6-methoxy-8-(4-amino-1-methylbutylamino)quinoline, PQ) is an antimalarial drug. PQ and other 8-aminoquinolines have been shown to be toxic to some sensitive individuals with G6PD deficient erythrocytes. Despite the widespread use of the drug, the metabolism of PQ is not well understood.

Some possibilities were suggested by the early work on the

metabolism of pamaquine by Brodie and Udenfriend (1950). From studies in dogs, they speculated that there was formation of a 5-hydroxy metabolite which reversibly produced a quinonimine capable of oxidizing hemoglobin to methemoglobin. Josephson et al. (1951) isolated a quinone of pamaquine formed by chickens and suggested that this metabolite may be the active agent against the malaria parasites.

In early studies in man, Zubrod (1948) observed that the highest plasma concentration of unmetabolized primaquine in patients was reached after 6 hours, then fell rapidly to virtually zero after 24 hours. Other investigators also found that PQ, pamaquine, and pentaquine underwent rapid biotransformation in the rhesus monkey (Hughes and Schmidt, 1950; Smith, 1956). Only relatively small amounts of the unmetabolized PQ were found in the urine (Smith, 1956). The plasma level of metabolites of PQ and pentaquine exceeded that of the parent compounds (Hughes and Schmidt, 1950).

More recently Baty et al. (1975) found rapid metabolism of PQ in man by measuring the PQ level in blood and the urinary excretion after oral administration of PQ to male subjects. They found PQ in red cells but not in plasma extracts. Using gas chromatography and mass spectrometry, they found that PQ underwent N-dealkylation to form a 6-methoxy-8-aminoquinoline derivative.

In studying the tissue distribution of PQ in rat, Holbrook et al. (1981) showed that concentration of PQ metabolites exceeded that of PQ itself and that unmetabolized PQ had a relatively short half-life, 4.0 hour in lung, 1.7 - 1.9 hour in blood, spleen, kidney and heart; and

1.2 hour in liver. The tissue concentrations of unmetabolized PQ at 3 hours after PQ injection were in the order: lung > kidney, spleen, liver > heart > brain > blood. They could not demonstrate any ^{3}H-labeled metabolite with the quinoline structure in either extracts of rat tissues or after the *in vitro* incubation of PQ with rat hepatic microsomes in the presence of NADPH under air.

The investigation of Strother *et al.* (1981) in dogs given PQ indicated that the PQ is metabolized rapidly or distributed from the blood to other parts of the body. These investigators observed that four or five metabolites of PQ are present in the urine of dog within five hours after injection, some of which cause methemoglobin formation in dog and normal or G6PD-deficient human erythrocytes. In these experiments, using thin-layer chromatography and comparing with model metabolites, Strother *et al.* (1981) identified 6-hydroxy-8-(4-amino-1-methylbutylamino)quinoline (6HPQ), 5-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline (5HPQ), and unmetabolized PQ from urinary extracts of dogs. There was about 46.1%, 9.5% and 19.7% of 5HPQ, 6HPQ and PQ, respectively. Strother *et al.* (1983) identified unmetabolized PQ and 5,6-dihydroxy-8-(4-amino-1-methylbutylamino)quinoline (5H6DPQ) from an incubation mixture of PQ and mouse liver microsomes in the presence of an NADPH-generating system.

Recently, Baker *et al.* (1982) have identified a carboxylic acid metabolite (8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline) in plasma from rat after PQ intravenous injection using high-performance liquid chromatographic, gas chromatographic, and mass spectral

methods. They also found that the concentration of metabolites is higher than that of PQ itself in the plasma except for the first few minutes after PQ injection.

Magon et al. (1981) reported that the various hemolytic responses among G6PD-deficient subjects to PQ and other hemolytic drugs may be due to the variation of hydroxylation of these compounds via the cytochrome-P450 mixed function oxidase system.

These findings suggest the possibility that metabolites of PQ are responsible for the therapeutic as well as the toxic effects of PQ. In any event, it is important to identify the structure of PQ metabolite(s) which is responsible for the antimalarial and toxic effects. Modification of the structure of PQ and its metabolite(s) to develop a new drug with high therapeutic index and diminished hemolytic effect in sensitive subjects may improve malaria chemotherapy.

Mechanism of Hemolysis by Primaquine

In the decade of the 1950's, many investigators studied the hemolytic effect of PQ on sensitive subjects. The hemolysis was found to be due to an intrinsic defect of the red cells of individuals sensitive to PQ (Dern et al., 1954a). Using ^{51}Cr-labelled PQ-sensitive erythrocytes, Dern et al. (1954b) found that the acute hemolysis after PQ administration in 9 daily doses of 30 mg base was self-limited; the blood counts returned to normal spontaneously even when drug administration was continued. Except for the presence of Heinz bodies in the red cells of sensitive subjects just before and at

the beginning of hemolysis, Beutler et al. (1954a) could not detect any significant differences between normal and sensitive cells either before or during PQ administration. These investigators (Beutler et al., 1954b) later discovered that ^{59}Fe-labeled red cells from sensitive individuals were insensitive to the hemolytic effect of PQ when they were 8 to 21 days old. However, when the age of these red cells exceeded one-half of their life span, they were rapidly destroyed by PQ and certain aniline derivatives. Red cells contain a variety of enzyme systems which catalyze a large number of metabolic processes. Concentrations of many enzymes and coenzymes are decreased as the red cells become older (Ponder, 1951). This suggested that a deficiency in one of the enzymes in older red cells may play a role in PQ sensitivity. Furthermore, Dern et al. (1955) reported that PQ sensitivity was a manifestation of a multiple drug sensitivity. All the drugs they studied had oxidant properties. The studies of Beutler et al. (1955) indicated that PQ-sensitive erythrocytes carried on normal glycolysis but contained only about 60% of the normal amount of GSH. The finding by Carson et al. (1956) that the PQ-sensitive cells were deficient in glucose-6-phosphate dehydrogenase (G6PD) activity provided the first evidence for an explanation of these findings. The decreased supply of NADPH from the pentose phosphate shunt can slow the regeneration of GSH from GSSG (Beutler, 1978).

On the basis of the previously reported studies on PQ metabolism, Tarlov et al. (1962) suggested that the hemolytic effect of PQ may be due to an oxidant quinone, a degradative intermediate, which has

anticatalase effect. The quinone may undergo a reversible oxidation-reduction reaction and eventually be metabolized to a quinonimine.

Cohen and Hochstein (1964) found that PQ in high concentrations (1 mM) produced hydrogen peroxide and methemoglobin in normal erythrocytes. They proposed that this could be a mechanism for oxidation of GSH and ultimately hemolysis in G6PD-deficient erythrocytes. Internalization of the red cell membrane and formation of intracellular vacuoles were also observed after incubation of normal human erythrocytes with high concentrations of PQ by Ginn, Hochstein and Trump (1969). They suggested that this morphological change in the red cell membrane led to reduction in surface area and was significant in red cell destruction.

Following the lead of Brodie and Udenfriend (1948) with pamaquine, Fraser and Vesell (1968) found that metabolites formed from PQ administered to dogs produced red cell damage and hemolysis; this was subsequently confirmed and extended by Strother et al. (1975, 1981). Fraser and Vesell (1968) also found that 5,6-dihydroxy derivative of pentaquine and 5,6-dihydroxy-8-aminoquinoline were considerably more active than PQ in oxidizing reduced glutathione and producing methemoglobin in both normal and G6PD-deficient human erythrocytes. They suggested that metabolites of PQ rather than PQ itself may be responsible for producing the damage to the red cell. Subsequently they (Fraser et al., 1971) also showed that these compounds oxidized GSH more readily in an older population of G6PD-deficient cells (with low levels of G6PD) than in a younger population

of G6PD-deficient cells (with high levels of G6PD).

Another possibility was raised by the finding of Wittles (1970) that modification of phospholipid metabolism in human red cell membranes could be produced by PQ. He suggested that this might be another possible mechanism in drug-induced hemolysis.

The production of superoxide radicals by high concentrations of primaquine has been recently reported by Summerfield and Tudhope (1978) and confirmed by Fraser et al. (1981). Hydrogen peroxide formed from the superoxide radical led to methemoglobin formation. Again Fraser et al. (1981) found that hydroxylated derivatives of PQ were much more active than PQ in producing superoxide anion or hydrogen peroxide.

On the basis of some of the above experimental observations the following possible events seem likely in the production of hemolysis by PQ administration in a G6PD-deficient individual: (1) PQ is converted to reactive metabolites by the microsomal mixed-function oxidase system; (2) superoxide radicals or hydrogen peroxide produced by PQ metabolites or its nonenzymatic degradation products, may mediate hemoglobin oxidation, glutathione oxidation, and oxidative damage to the erythrocyte membrane; (3) however the glutathione peroxidase system may protect against cell damage by peroxide at the expense of reduced glutathione (Chance, Sies and Boveris, 1979); (4) G6PD-deficient erythrocytes are less capable of restoring normal reduced glutathione content due to the limited activity of the pentose phosphate pathway (Carson et al., 1956; Tarlov et al., 1962); (5) disruption of the cell membrane structure may lead to electrolyte

leakage and imbalances across the membrane (Judah et al.,1970); (6) the damaged cells may then be eliminated by intravascular hemolysis or removed by the reticuloendothelial system.

As will be discussed in Chapter 2, recent studies with other drugs suggest that covalent binding of reactive metabolites may play a significant role in many different forms of drug toxicity. Also, as will be discussed in Chapter 3, lipid peroxidation may be an important factor in damage to membranes by drugs. The investigations reported here were undertaken in order to explore the possible role of these two phenomena in the mechanism of the hemolytic effect of PQ in relation to the current evidence summarized above.

CHAPTER 2: COVALENT BINDING OF PRIMAQUINE TO MOUSE LIVER MICROSOMES

Introduction

Primaquine (PQ, an 8-aminoquinoline, has been used widely in radically curative treatment of exoerythrocytic vivax malaria since the 1940s. It has long been known that after large doses (30 mg) of PQ or in sensitive individuals, severe hemolytic anemia, leukopenia and methemoglobinemia may be produced.

Fraser and Vesell (1968) found that the mechanical, but not the osmotic, fragility of G6PD-deficient erythrocytes was increased more than that of normal erythrocytes by hydroxylated model metabolites of pentaquine and acetanilid. These compounds also increased the methemoglobin content, decreased the reduced glutathione content and decreased the NADPH/NADP ratio more markedly in G6PD-deficient red cells than in normal cells; PQ at the same concentration was much less active.

Many drugs and foreign compounds can be converted in the animal body to inert or active metabolites. The liver is the major organ of drug metabolism. Drugs are usually metabolized to more polar compounds which can be more readily excreted. However, chemically reactive metabolites may be formed which subsequently induce severe toxicities by uncoupling integrated biochemical processes in cells or by combining covalently with tissue macromolecules. Toxicities including hemolytic anemia, methemoglobinemia, mutagenesis, cellular necrosis, hypersensitivity reactions, blood dyscrasias, and fetotoxicities, are known to be mediated by chemically reactive

metabolites (Gillette et al., 1974).

A wide variety of chemicals with unrelated structures, such as N-alkyl-N-nitroso compounds, ethionine, urethane, polycyclic aromatic hydrocarbons, aromatic amines, 4-nitroquinoline N-oxide and 2-acetylaminofluorene, can be converted to alkylating, arylating agents or reactive metabolites and react with crucial cellular macromolecules (DNA, RNA or proteins). The subsequent covalent interaction of these compounds with tissue proteins may result in carcinogenesis (Miller and Miller, 1947, 1952, 1966; Miller, 1970; Magee and Barnes, 1967; Farber, 1963).

Judah et al. (1970) suggested that the hepatic injury caused by some hepatotoxins might be mediated by their chemically reactive metabolites which bind to tissue macromolecules covalently. Since then many investigators have found that acetaminophen as well as phenacetin is converted to toxic metabolites by hepatic microsomal enzymes. The metabolites depleted hepatic reduced glutathione and were covalently bound to tissue protein. The severity of hepatic necrosis paralleled the magnitude of covalent binding (Mitchell et al., 1975). The hepatic toxicity of acetaminophen was enhanced by a liver microsomal enzyme inducer (phenobarbital) and decreased by microsomal enzyme inhibitors (piperonyl butoxide, SK&F-525A, cobaltous chloride) (Mitchell et al., 1973a, b; Jollow et al., 1973; Potter et al., 1973). Similar effects of enzyme inducers and inhibitors on the hepatic toxicity of carbon tetrachloride and chloroform (Judah et al., 1970), acetylisoniazid; acetylhydrazine and iproniazid (Nelson et

al., 1977) have also been reported.

It seems possible that chemically reactive metabolites of PQ could leave the liver after depletion of its reduced glutathione and bind to red cell membrane protein covalently as do bromobenzene metabolites to lung and heart muscle tissue proteins (Reid et al., 1973).

In the investigation we are reporting here, we have tested the hypothesis that PQ metabolites formed by mouse liver microsomes may bind to microsomal protein covalently. We have also investigated the possible modification of this binding by inducers and inhibitors of microsomal enzymes. The possibility that formation of these metabolites may produce a decrease of mouse liver and erythrocyte reduced glutathione content following administration of PQ to mice was also studied.

Materials and Methods

Chemicals

Tritiated primaquine (^{3}H-PQ), labelled in the ring system, was obtained from New England Nuclear Corporation and recrystallized in the laboratory with nonlabelled PQ to obtain a specific activity of 0.137 mCi/mmol. ^{14}C-PQ was supplied by the Special Programme for Research and Training in Tropical Diseases of the World Health Organization. It was recrystallized with nonlabelled PQ to obtain a specific activity of 1.03 Ci/mmol. The purity of the radioactive compounds was greater than 90% as verified by thin layer chromatography with methanol-benzene (2:1) on silica gel (Strother et al., 1981).

Primaquine diphosphate was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (Type XII) (G6PD), 1-cysteine HCl, glutathione (reduced form), phenobarbital, hexobarbital and bovine serum albumin (Fraction V) were obtained from Sigma Chemical Company, St. Louis, MO. Magnesium chloride ($MgCl_2.6H_2O$) was supplied by J. T. Baker Chemical Company, Phillipsburg, NJ. Beta-diethylaminoethyldiphenylpropylacetate hydrochloride (SK&F-525A) was supplied by Smith Kline and French Laboratories, Philadelphia, PA. Metyrapone (MTP) was a gift obtained from CIBA Pharmaceutical Co., Summit, NJ.; piperonyl butoxide (PPB) was obtained from K & K Laboratories, Inc., Plainview, NY. All other chemicals were of highest purity available.

Preparation of Microsomes

Male Swiss-Webster mice (Simonsen Laboratory, Gilroy, CA) weighing 22-27 g were given regular Purina Lab Chow and water *ad libitum*. They were killed by cervical dislocation, the livers were removed immediately, blotted on absorbent paper and placed in a volume of ice cold 1.15% KCl-0.025 M Tris buffer, pH 7.35, equal to twice the weight of tissue. Microsomes were prepared in the same buffer, as described by Peters and Strother (1971). The protein content was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the reference standard. Cytochrome P450 content was measured as described by Peters and Fouts (1970).

Incubation Mixture

Tritiated primaquine or ^{14}C-PQ, in 0.25 M Tris buffer (pH 7.35), at concentrations indicated in "Results", was incubated at 37°C with mice liver microsomes (10 mg) and 1.0 ml of 0.25 M Tris buffer (pH 7.35) containing an NADPH-generating system: NADP (4 μmol), G6P (25 μmol), $MgCl_2$ (30 μmol), and G6PD (2 units). Control flasks received 1.0 ml of 0.25 M Tris buffer (pH 7.35) only. The final volume of incubation mixture was brought to 5.0 ml with 1.15% KCl-0.025 M Tris buffer (pH 7.35) and distilled water. The reactions were carried out under air in a Dubnoff shaking incubator and were stopped by adding 1.0 ml of 10% trichloroacetic acid (TCA) at the desired time. In some experiments, SK&F-525A, MTP, PPB, l-cysteine HCl or reduced glutathione (Tredger et al., 1981) at concentrations indicated in "Results", was added to the incubation mixture to study their effects on covalent binding of PQ to mice liver microsomes.

In some studies, microsomes were prepared from mice pretreated in several ways. With phenobarbital (60 mg/Kg i.p. twice daily in normal saline for 3 days) the mice were killed 24 hours after the last dose). SK&F-525A (50 mg/Kg i.p. in normal saline) was administered 40 minutes before sacrifice (Cook et al., 1954) while PPB (1,360 mg/Kg i.p. in corn oil) was given 30 minutes before sacrifice (Mitchell et al., 1973a). Control mice received normal saline or corn oil for similar periods.

Determination of Covalent Binding of PQ to Mice Liver Microsomes

The incubation mixtures were transferred to 13 ml centrifuge tubes and centrifuged at 500 x g (International Refrigerated Centri-

fuge, Model PR6) for 15 minutes at room temperature. The supernatant was discarded, and the protein precipitate was resuspended in 3.0 ml of 10% TCA and mixed on a Vortex shaker for 3 minutes. The tubes were centrifuged again at 500 x g for 10 minutes, and the protein pellets were washed again with 3.0 ml 10% TCA. The protein was then resuspended in 2 ml 80% methanol, mixed for 3 minutes, centrifuged at 1000 x g for 10 minutes at room temperature and the supernatant discarded. This extraction was repeated until no further radioactivity could be removed (Jollow et al., 1973). In our experiments, we routinely extracted the precipitated protein 9 times with methanol even though 6 to 7 extractions were sufficient to remove all the reversibly bound radiolabel. The extracted microsomal protein was dissolved in 1.0 ml (1N NaOH) and an aliquot of 20 µl was added to 10.0 ml Cytoscint scintillation cocktail (West Chem Products, San Diego, CA) and counted in a Beckman LS-250 scintillation counter for radioactivity. To ensure that the radiolabel was bound to microsomal protein and not to lipid, some samples were extracted 3 times with 2 ml hexane before extracting with 80% methanol.

In vivo Covalent Binding

Mice were sacrificed 60 minutes after receiving varying doses of ^{3}H-PQ or ^{14}C-PQ and microsomes were prepared as in the in vitro studies. To 1.0 ml of each microsome homogenate, 2.0 ml of 10% TCA was added to precipitate the protein. The protein pellet was washed twice with 10% TCA and extracted with 80% methanol for 9 times as described above. An aliquot of 100 µl was counted for radioactivity. In other experiments, covalent binding of PQ to microsomes from mice

pretreated with phenobarbital, SK&F-525A, and PPB as described above were also studied.

Primaquine Effects on Tissue Glutathione Levels

Mice were injected with PQ (80 mg/Kg, i.p.) and sacrificed by cervical dislocation at various times after PQ administration. Blood was collected by cardiac puncture with heparin as anticoagulant. The blood was centrifuged at 1000 x g for 15 minutes. An aliquot of the packed erythrocytes was transferred to a centrifuge tube and lysed with 10 times its volume of distilled water. Five percent trichloroacetic acid--0.02 M disodium ethylenediaminetetraacetic acid solution (TCA-EDTA) in a volume equal to that of the original red cells was added. Livers were removed from the mice immediately after cervical dislocation, rinsed in ice cold normal saline, blotted and weighed before transferring to homogenization tubes. A sufficient volume of TCA-EDTA was added to the liver to give a 20% w/v homogenate. The tissue was homogenized using a glass mortar and a motor driven teflon pestle. The homogenates and lysed red blood cells in TCA-EDTA were centrifuged at 1000 x g for 15 minutes, 0.4 ml of the protein free supernatant was mixed with 4.55 ml of a 0.01 M sodium phosphate buffer, pH 8.0. The blood samples were filtered through Whatman No. 1 filter paper. To each solution, 0.05 ml of 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid((DTNB, Ellman's reagent) in absolute methanol was added and absorbance of the resultant yellow colored solution against a reagent blank was determined in a Gilford spectrophotometer at 412 nm within 2 minutes (Sedlak and Lindsay, 1968; Olson et al., 1980). Control values were also obtained when no PQ was administered. Data

were expressed as percentage of the control values. Glutathione (reduced form) was used as the reference standard.

Statistics

Data were expressed as mean ± S.E.M. The significance of the differences between means was determined by Students' t-test. Statistical differences were considered to be significant with $p < 0.05$.

Results

As can be seen in Figure 1, considerable covalent binding of tritiated compounds to mouse liver microsomes occurred when they were incubated with ^{3}H-PQ. The reaction was linear for 90 minutes with or without the presence of an NADPH-generating cofactor system. The amount of binding in the absence of cofactors was subtracted from that in the presence of cofactors to provide the net covalent binding probably attributable to metabolite(s). There was a steady and highly significant increase in net binding of tritiated metabolites during the first 90 minutes of the incubation; the net binding began to decline slowly after 150 minutes of incubation. In the absence of cofactors, PQ itself or spontaneous breakdown products of PQ apparently became bound to microsomal protein.

The amount of the net covalent binding of primaquine depended on the concentration of mouse liver microsomal protein, as shown in Figure 2. The net binding was linear up to a concentration of 10 mg protein per 5 ml of incubation mixture and then decreased with 20 mg protein per 5 ml. Therefore, in studying the binding reactions, we

routinely used a microsomal protein concentration of 10 mg in 5 ml of incubation mixture.

The possibility that the radioactivity in the methanol-extracted microsomes was partially attributable to primaquine still dissolved in lipid was tested by extracting first with hexane, a relatively nonpolar organic solvent. As shown in Table 1, three extractions with hexane followed by nine extractions by 80% methanol did not alter the binding of radioactive material from ^{3}H-PQ to the microsomes as compared to that found with nine extractions with 80% methanol alone.

The effect of the concentration of ^{3}H-PQ on the extent of covalent binding in the presence and absence of cofactors was studied. As shown in Table 2, covalent binding occurred to a virtually similar extent with or without cofactors at a ^{3}H-PQ concentration of 1 mM, whereas at low concentrations of 0.001 mM or 0.01 mM, binding was increased significantly by the presence of the cofactors. This suggests that at these low concentrations of PQ the increase in covalently bound compounds in the presence of cofactors was most probably due to metabolites of PQ. As shown in Figure 3, an approximately linear relationship was found when the amount of ^{3}H bound to the microsomal protein is plotted against the logarithm of the concentration of PQ in the incubation mixture over the range of 0.005 to 0.05 mM. On the basis of these findings a concentration of ^{3}H-PQ of 0.01 mM was used in most of the other studies reported here.

In order to further establish that the net binding was attributable to metabolism of PQ in the presence of the NADPH-generating system, we studied the effects of an inducer of cytochrome P450 and

inhibitors of its function. Using microsomes in which the cytochrome P450 content was increased 75% by phenobarbital pretreatment, net covalent binding *in vitro* was enhanced markedly over a wide range of primaquine concentrations, as shown in Figure 4. On the other hand, when the cytochrome P450 inhibitors, SK&F-525A (0.1 mM) or metyrapone (1 mM), were added to the incubation mixture (as described in "Methods") the net covalent binding was inhibited almost completely (Figure 4). These results indicate that the net covalent binding of ^{3}H to microsomal protein was due to metabolite(s) of primaquine produced by the action of the microsomal mixed function oxidase in the presence of the NADPH-generating system.

Figure 5 shows a double-reciprocal plot of net covalent binding *in vitro* of ^{3}H-PQ metabolite(s) to microsomes prepared from control and phenobarbital pretreated mice. The kinetic parameters were determined by calculation. The apparent Michaelis constant (Km) for both control and phenobarbital pretreated mice microsomes was 2.6×10^{-5} M. The maximum velocity (V_{max}) for microsomes from control mice was 1.1×10^{-2} nmoles/mg protein/minute while that for microsomes from phenobarbital pretreated mice was 1.7×10^{-2} nmoles/mg protein/minute. Phenobarbital pretreatment increased the rate of binding by almost 55% without changing the apparent Km.

Due to the possible lability of tritium on the PQ molecule, we also used ^{14}C-PQ in some binding studies. In Tables 3 and 4 the binding of ^{3}H-PQ is compared to that of ^{14}C-PQ by control and phenobarbital-pretreated microsomes and also by microsomes in the presence of SK&F-525A or metyrapone. The binding was somewhat less, but not

significant, with ^{3}H-PQ than with ^{14}C-PQ except for the net binding in the presence of metyrapone; however the results were qualitatively very similar. Since the supply of ^{14}C-PQ was quite limited it was necessary to use ^{3}H-PQ for most of the studies.

Piperonyl butoxide (PPB), another inhibitor of microsomal-drug metabolizing enzymes, also markedly inhibited the covalent binding of ^{3}H-PQ to mouse liver microsomal protein when it was added to the incubation mixture, as shown in Table 5. However, the *in vitro* covalent binding of ^{3}H-PQ to microsomes prepared from piperonyl butoxide (1360 mg/Kg, i.p.) or SK&F-525A (50 mg/Kg, i.p.) pretreated mice was only inhibited by 14% (Table 6) although there was a somewhat greater percentage decrease in cytochrome P450.

The effect of the sulfhydryl-containing compounds, cysteine and glutathione, at a concentration of 1 mM, on covalent binding of ^{3}H-PQ metabolites to mouse liver microsomal protein was also studied, with the results shown in Table 7. In these studies, covalent binding was measured in either the presence or the absence of G6PD in the cofactor mixture. Glutathione decreased the binding in the absence of G6PD but cysteine was without significant effect. In the presence of G6PD extensive binding occurred in the controls but both reagents inhibited the net covalent binding almost completely.

As shown in Tables 8 and 9, it was possible to demonstrate binding of ^{3}H-PQ to microsomal protein *in vivo*. When SK&F-525A or piperonyl butoxide was administered to mice intraperitoneally before the ^{3}H-PQ injection, there was no significant difference in covalent binding *in vivo* between controls and the pretreated mice. These doses

of SK&F-525A and piperonyl butoxide dramatically prolonged hexobarbital sleeping time in the mice indicating that these compounds indeed inhibited drug metabolism. Apparently the covalent binding of ^{3}H-PQ *in vivo* may not involve metabolism.

Figure 6 shows a linear relationship between the dose of ^{3}H-PQ and its *in vivo* covalent binding to mouse liver microsomes. At a dose of 10 mg/Kg, ^{14}C-PQ also bound covalently to microsomes to an extent similar to that seen with the tritiated isotope. However, the *in vivo* covalent binding of ^{14}C-PQ to microsomes of phenobarbital pretreated mice was significantly less than that to microsomes of control mice. In preliminary studies a similar phenomenon also occurred with different doses of ^{3}H-PQ (data not shown). Apparently increased metabolism of PQ *in vivo* may decrease covalent binding.

The possibility that reactive metabolites of PQ may oxidize GSH *in vivo* was examined by measuring the reduced glutathione content of mouse liver and erythrocytes after injection of 80 mg/Kg of PQ. Figure 7 shows that hepatic GSH was significantly depleted at 60 minutes after the time of injection; the normal level of GSH was regained at 4 hours after primaquine administration. The GSH content of erythrocytes was more markedly decreased than that of the liver after 60 minutes and took 8 hours to return to normal after the primaquine injection, as shown in Figure 8.

Discussion

In terms of criteria used by previous investigators (Jollow *et*

al., 1973; Mitchell et al., 1973a, b; Potter et al., 1973), the data in Tables 1 and 2 and Figures 1 and 6 clearly show covalent binding of PQ to microsomal protein both in vitro and in vivo. The failure of the pre-extraction with hexane (Table 1) to decrease the binding found after extraction with methanol appears to rule out the possibility that significant amounts of the bound radioactivity are due to ^{3}H-PQ dissolved in the lipids of the microsomes. At a concentration of 0.01 mM PQ, the net binding of radioactivity due to the presence of the NADPH-generating system accounts for over 80% of the total binding, at least for the first 90 minutes of the incubation (Figure 1). Conversion of PQ to reactive metabolites by the mixed function oxidase system is the most obvious interpretation of this result. At higher concentrations the net binding attributable to the presence of the NADPH-generating system decreased (Table 2); since PQ itself is relatively unreactive, nonenzymatic breakdown of PQ to reactive products probably accounts for the binding seen in the controls without cofactors.

The marked increase in covalent binding in vitro to microsomes prepared from mice pretreated with phenobarbital and containing increased levels of cytochrome P450 (Table 3, Figures 4 and 5) also clearly points to the role of metabolite(s) in the covalent binding (Mitchell et al., 1973a, b; Potter et al., 1973). The marked decrease of the net covalent binding produced by the addition of the mixed function oxidase inhibitors, SK&F-525A, metyrapone, and piperonyl butoxide (Mitchell et al., 1973b) shown in Tables 4 and 5 and Figure 4, again supports the interpretation that metabolites play a major role in the process of covalent binding in vitro at low concentrations (0.01 mM)

of PQ.

In contrast to the increased covalent binding seen *in vitro*, phenobarbital pretreatment decreased covalent binding of PQ *in vivo* (Figure 6). McLean and Marshall (1971) reported that phenobarbital diminished rather than increased the carcinogenic effects of aflatoxin in rats. Benedict *et al.* (1973) suggested that it is possible that low levels of microsomal enzymes are sufficient for drug metabolism, whereas enhanced drug metabolism by inducers of drug metabolism enzymes may further convert some metabolites to inert derivatives. Another microsomal enzyme inducer, 3-methylcholanthrene (3-MC), has been found to potentiate acetaminophen-induced hepatic necrosis (Mitchell *et al.*, 1973a, b) but protect against carbon tetrachloride-induced (Suarez *et al.*, 1971) and bromobenzene-induced (Reid *et al.*, 1971) hepatic necrosis. It is probable that besides inducing metabolic enzymes to produce more reactive metabolites and thus enhance toxicities, these inducers stimulate an alternate pathway of metabolism which may compete with the toxic route, or a detoxification one (such as conjugation) to convert the toxic metabolites to innocuous derivatives. Such events may also account for our results with PQ *in vivo*.

Again, in contrast to the *in vitro* results (Tables 4 and 5, Figure 4), the effect of the inhibitors, SK&F-525A and PPB, on covalent binding *in vivo* was not significant (Tables 8 and 9) even though the cytochrome P450 mixed function oxidase was indeed inhibited, as shown by the prolongation of hexobarbital sleeping time. It is possible that the level of enzyme activity remaining *in vivo* in the presence of inhibitors is sufficient to produce the relatively lower levels of

covalent binding found *in vivo*. Also other *in vivo* detoxification pathway(s) may be inhibited, thus increasing the supply of reactive metabolite(s) of PQ which bind to microsomal protein. Alternatively, the binding *in vivo* in the presence of the inhibitors may be due to reactive products formed from PQ by nonenzymatic processes.

Our data show that cysteine and glutathione almost completely inhibited the covalent binding of PQ metabolite(s) to mouse hepatic microsomal protein *in vitro* (Table 7). Thiol groups such as those of GSH can combine with reactive metabolites to form S-alkyl or S-aryl derivatives (Waley, 1966) thus protecting the thiol groups on tissue proteins against covalent binding. Administration of PQ to mice depleted liver and erythrocyte GSH levels (Figures 7 and 8) as would be expected if reactive metabolites are produced *in vivo* and react with the GSH. This correlates well with the report by Fraser and Vesell (1968) that the GSH content of G6PD-deficient erythrocytes can be decreased by incubation with model metabolites of pentaquine. The time course of covalent binding to microsomes *in vitro* and depletion of GSH *in vivo* show interesting parallels (cf. Figures 1 and 7); studies of the time course of covalent binding *in vivo* would be needed to establish this relationship more closely. These results parallel those of other investigators who have shown that sulfhydryl-containing compounds such as glutathione and cysteine prevent the arylation of hepatic macromolecules by toxic metabolites of acetaminophen *in vitro* and *in vivo* without inhibiting the metabolism of the drug (Jollow *et al.*, 1973; Mitchell *et et.*, 1973b; Tredger *et al.*, 1981). Furthermore, pretreatments that affected acetaminophen-induced hepatic

toxicity also altered the depletion of hepatic glutathione (Mitchell et al., 1973b). Olson et al. (1980) have shown that doxorubicin, a potent anticancer drug, depleted GSH levels in mouse liver, erythrocytes and heart but induced only dose-dependent cardiotoxicity.

Even though our data indicate that PQ metabolite(s) and/or breakdown products can covalently bind to mouse liver microsomal protein in vivo as well as in vitro, we are not aware of any evidence of hepatic damage following administration of PQ. As suggested by Mitchell et al. (1975), many drugs can be converted to active metabolites and become covalently bound to microsomal protein, but yet this fact does not necessarily predict the occurrence of toxicity in vivo. However, we (Chau et al., 1983) have found that reactive products from PQ may covalently bind to erythrocytes, their membranes and hemoglobin, thus suggesting that covalent binding of such products could damage erythrocytes and thus play a role in PQ-induced hemolytic anemia in G6PD-deficient individuals.

Table 1. Covalent binding of 0.01 mM ^{3}H-primaquine to mouse liver microsomes *in vitro* by extraction with hexane and 80% methanol or with 80% methanol alone

	nmoles bound/mg protein/60 minutes[a]		
Extraction with	No Cofactors	With Cofactors	Net Bound
Hexane and 80% methanol	0.04 ± 0.003	0.20 ± 0.01	0.16 ± 0.01
80% methanol	0.04 ± 0.003	0.21 ± 0.03	0.17 ± 0.03

[a]Values shown are the mean and standard error of 3 to 4 experiments.

Table 2. Covalent binding of 0.001 mM to 1 mM ^{3}H-primaquine (^{3}H-PQ) to mouse liver microsomal protein *in vitro* with or without cofactors

	nmoles bound/mg protein/30 minutes[a]		
^{3}H-PQ Concen.	No Cofactors	With Cofactors	Net Bound
1 mM	1.98 ± 0.22	2.11 ± 0.13	0.14 ± 0.14
0.1 mM	0.23 ± 0.03	0.55 ± 0.08[b]	0.37 ± 0.06
0.01 mM	0.03 ± 0.001	0.13 ± 0.002[c]	0.09 ± 0.003
0.001 mM	0.01 ± 0.0003	0.03 ± 0.003[c]	0.02 ± 0.003

[a]Values shown are the mean and standard error of 3 to 5 experiments.

[b]Significantly different from the binding without cofactors at $p < 0.010$.

[c]Significantly different from the binding without cofactors at $p < 0.005$.

Table 3. Covalent binding of 0.01 mM radiolabeled primaquine to control and phenobarbital pretreated mouse liver microsomal protein *in vitro*

		nmoles bound/mg protein/30 minutes[a]		
Pretreatment	Form of PQ	No Cofactors	With Cofactors	Net Bound
Control	^{3}H-PQ	0.03 ± 0.001	0.13 ± 0.002	0.09 ± 0.003
Control	^{14}C-PQ	0.05 ± 0.01	0.16 ± 0.01	0.11 ± 0.01
Phenobarbital[b]	^{3}H-PQ	0.04 ± 0.001	0.19 ± 0.01	0.15 ± 0.01[c]
Phenobarbital[b]	^{14}C-PQ	0.04 ± 0.02	0.24 ± 0.01	0.20 ± 0.02[c]

[a]Values shown are the mean and standard error of 3 to 4 experiments.

[b]Mice received 60 mg/Kg of phenobarbital twice daily for 3 days and were sacrificed 24 hours after the last dose. The cytochrome P450 content of control mice liver microsomes was 0.76 ± 0.004; of phenobarbital pretreated mice liver microsomes was 1.58 ± 0.12 nmoles/mg protein.

[c]Significantly different from control at $p < 0.005$.

Table 4. Effect of 0.1 mM SK&F-525A and 1 mM metyrapone (MTP) on covalent binding of 0.01 mM primaquine (PQ) to mouse liver microsomal protein *in vitro*

		nmoles bound/mg protein/30 minutes[a]			
Drug	Form of PQ	No Cofactors	With Cofactors	Net Bound	% Inhibition
Control	^{3}H-PQ	0.03 ± 0.001	0.13 ± 0.002	0.09 ± 0.03	
Control	^{14}C-PQ	0.05 ± 0.01	0.16 ± 0.01	0.11 ± 0.01	
SK&F-525A	^{3}H-PQ	0.02 ± 0.001	0.03 ± 0.002	0.01 ± 0.001[b]	91%
SK&F-525A	^{14}C-PQ	0.04 ± 0.01	0.05 ± 0.01	0.01 ± 0.003[b]	92%
MTP	^{3}H-PQ	0.07 ± 0.01	0.08 ± 0.03	0.02 ± 0.02[b]	83%
MTP	^{14}C-PQ	0.09 ± 0.01	0.09 ± 0.01	0.002 ± 0.001[b]	98%

[a]Values shown are the mean and standard error of 3 to 5 experiments.

[b]Significantly different from control at $p < 0.001$

Table 5. Effect of piperonyl butoxide (PPB) at 0.5 mM and 1 mM on covalent binding of 0.01 mM ^{3}H-primaquine to mouse liver microsomal protein *in vitro*

	nmoles bound/mg protein/30 minutes[a]			
Drug	No Cofactors	With Cofactors	Net Bound	% Inhibition
Control	0.05 ± 0.003	0.11 ± 0.002	0.06 ± 0.002	
PPB, 0.5 mM	0.02 ± 0.001	0.04 ± 0.002	0.01 ± 0.001[b]	80%
PPB, 1 mM	0.02 ± 0.001	0.03 ± 0.001	0.01 ± 0.002[b]	83%

[a]Values shown are the mean and standard error of 3 experiments.

[b]Significantly different from control at $p < 0.001$

Table 6. Effects of pretreatments on covalent binding of 0.01 mM ^{3}H-primaquine to mouse liver microsomal protein *in vitro*

Source of Microsomes	nmoles bound/mg protein/30 minutes[a] No Cofactors	With Cofactors	Net Bound	% Inhibition
Normal saline pretreated[b]	0.07 ± 0.01	0.14 ± 0.02	0.07 ± 0.01	
SK&F-525A pretreated[c]	0.07 ± 0.01	0.13 ± 0.01	0.06 ± 0.01	14%
Corn oil pretreated[d]	0.05 ± 0.004	0.15 ± 0.01	0.10 ± 0.01	
Piperonyl butoxide pretreated[e]	0.03 ± 0.002	0.12 ± 0.01	0.09 ± 0.02	14%

[a]Values shown are the mean and standard error of 3 to 5 experiments.

[b]Control mice received 0.01 ml/gm of 0.9% NaCl (i.p.) 40 minutes before sacrifice. Cytochrome P450 content was 0.79 ± 0.02 nmole/mg protein.

[c]SK&F-525A pretreated mice received 50 mg/Kg of SK&F-525A in 0.9% NaCl (i.p.) 40 minutes before sacrifice. Cytochrome P450 content was 0.67 ± 0.04 nmole/mg protein.

[d]Control mice received 0.01 ml/gm of corn oil (i.p.) 30 minutes before sacrifice. Cyrochrome P450 content was 0.84 ± 0.07 nmole/mg protein.

[e]Piperonyl butoxide pretreated mice received 1,360 mg/Kg of PPB in corn oil (i.p.) 30 minutes before sacrifice. P450 content was 0.59 ± 0.08 nmole/mg protein.

Table 7. Effect of sulhydryl-containing compounds on covalent binding of 0.01 mM ^{3}H-primaquine to mouse liver microsomal protein *in vitro*[a]

Drug	nmoles bound/mg protein/30 minutes[b]			% Inhibition
	Cofactors Without G6PD	Cofactors With G6PD	Net Bound	
Control	0.06 ± 0.01	0.21 ± 0.02	0.15 ± 0.02	
Cysteine, 1 mM	0.07 ± 0.02	0.07 ± 0.02	0.001 ± 0.002[c]	99%
Glutathione, 1 mM	0.03 ± 0.003	0.05 ± 0.04	0.02 ± 0.001[d]	88%

[a]The cofactors were added to the incubation mixture 5 minutes after incubation started.

[b]Values shown are the mean and standard error of 3 experiments.

[c]Significantly different from control at $p < 0.001$

[d]Significantly different from control at $p < 0.05$

Table 8. Effect of SK&F-525A on covalent binding of ^{3}H-primaquine (80 mg/Kg) to mouse liver microsomal protein *in vivo*

Pretreatment	nmoles bound/mg protein[a]
Control[b]	0.06 ± 0.01
SK&F-525A[c,d]	0.04 ± 0.01

[a]Values shown are the mean and standard error of 3 experiments.

[b]Control mice received 0.01 ml/gm of 0.9% NaCl 40 minutes before ^{3}H-primaquine (80 mg/Kg) was injected and the mice were sacrificed 60 minutes after PQ injection. The cytochrome P450 content was 0.85 ± 0.03 nmole/mg protein.

[c]SK&F-525A pretreated mice received 50 mg/Kg of SK&F-525A in 0.9% NaCl 40 minutes before ^{3}H-primaquine (80 mg/Kg) was injected and the mice were sacrificed 60 minutes after PQ injection. The cytochrome P450 content was 0.86 ± 0.02 nmole/mg protein.

[d]Hexobarbital sleeping time of SK&F-525A pretreated mice was significantly longer than that of control at $p < 0.001$. Control mice slept 43.0 ± 5.9 minutes and SK&F-525A pretreated mice slept over 24 hours.

Table 9. Effect of piperonyl butoxide (PPB) on covalent binding of ^{3}H-primaquine (80 mg/Kg) to mouse liver microsomal protein *in vivo*

Pretreatment	nmoles bound/mg protein[a]
Control[b]	0.06 ± 0.01
PPB[c,d]	0.07 ± 0.004

[a]Values shown are the mean and standard error of 3 experiments.

[b]Control mice received 0.01 ml/gm of corn oil (i.p.) for 30 minutes before ^{3}H-primaquine (80 mg/Kg) was injected and was sacrificed 60 minutes after PQ injection. The cytochrome P450 content was 0.83 ± 0.07 nmole/mg protein.

[c]Piperonyl butoxide pretreated mice received 1,360 mg/Kg of PPB in corn oil 30 minutes before ^{3}H-primaquine (80 mg/Kg) was injected and was sacrificed 60 minutes after PQ injection. The cytochrome P450 content was 0.24 ± 0.09 nmole/mg protein.

[d]Hexobarbital sleeping time of PPB treated mice was significantly longer than that of control at $p < 0.001$. Control mice slept 11.0 ± 2.5 minutes and PPB pretreated mice slept 138.8 ± 13.6 minutes.

Figure 1. Time course of covalent binding of 0.01 mM ^{3}H-primaquine to mouse liver microsomes. Each point is the mean and standard error of 3 to 6 experiments. ●———●, binding with cofactors; ■———■, binding without cofactors. (Each point is significantly different from binding with cofactors at the same incubation interval at $p < 0.005$.) o———o, net binding.

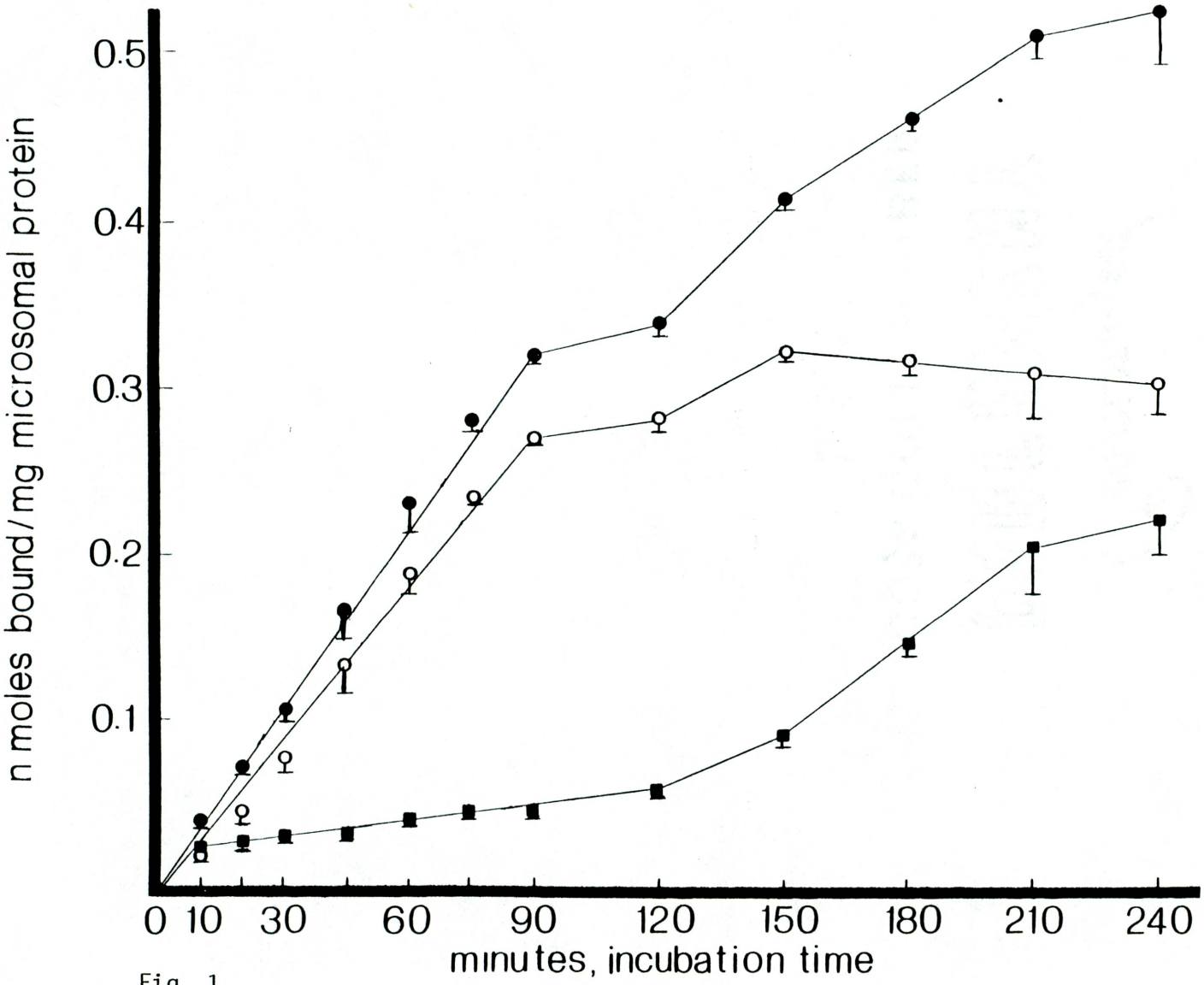
n moles bound/mg microsomal protein
0.5
0.4
0.3
0.2
0.1
0 10 30 60 90 120 150 180 210 240
minutes, incubation time

Fig. 1

Figure 2. Net covalent binding of 0.01 mM ^{3}H-primaquine to varying concentrations of mouse liver microsomal protein. Each point is the mean and standard error of 3 experiments.

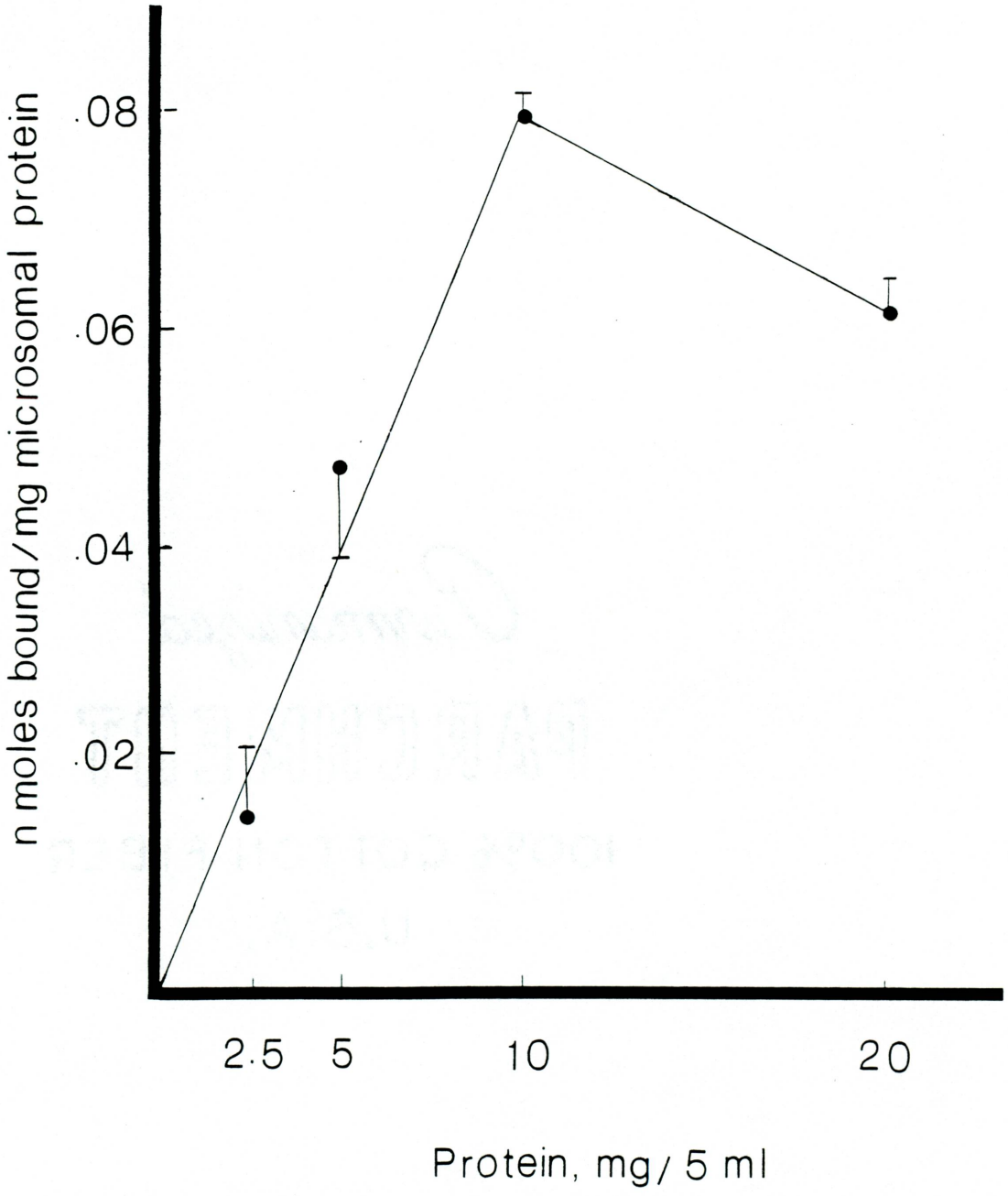
n moles bound/mg microsomal protein
.08
.06
.04
.02
2.5
5
10
20
Protein, mg/ 5 ml

Fig. 2

Figure 3. Covalent binding of different concentrations of ^{3}H-primaquine to mouse liver microsomal protein *in vitro*. Each point is the mean and standard error of 3 to 5 experiments. ●——●, binding with cofactors; ■——■, binding without cofactors; ○——○, net binding.

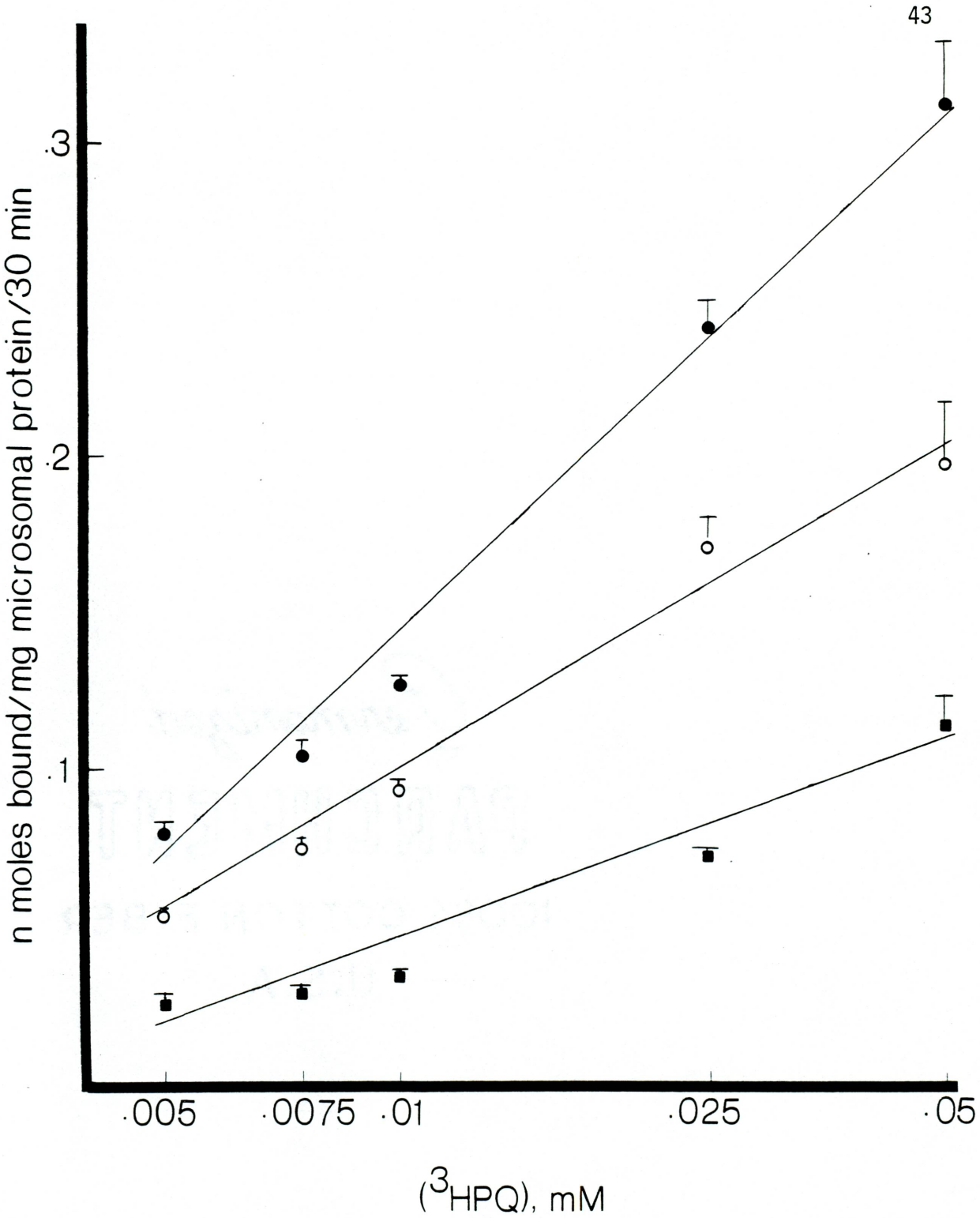
n moles bound/mg microsomal protein/30 min
.3
.2
.1
.005
.0075
.01
.025
.05
(^{3}HPQ), mM

Fig. 3

Figure 4. Effects of cytochrome P450 inducer and inhibitors on net covalent binding of 0.005 mM to 0.05 mM ^{3}H-primaquine to mouse liver microsomal protein *in vitro*. Each point is the mean and standard error of 3 to 5 experiments. ○——○, net binding to microsomes from control mice; ●——●, net binding to microsomes from phenobarbital pretreated mice; □——□, net binding to microsomes from control mice in the presence of 0.1 mM SK&F-525A; ■——■, net binding to microsomes from control mice in the presence of 1 mM metyrapone.

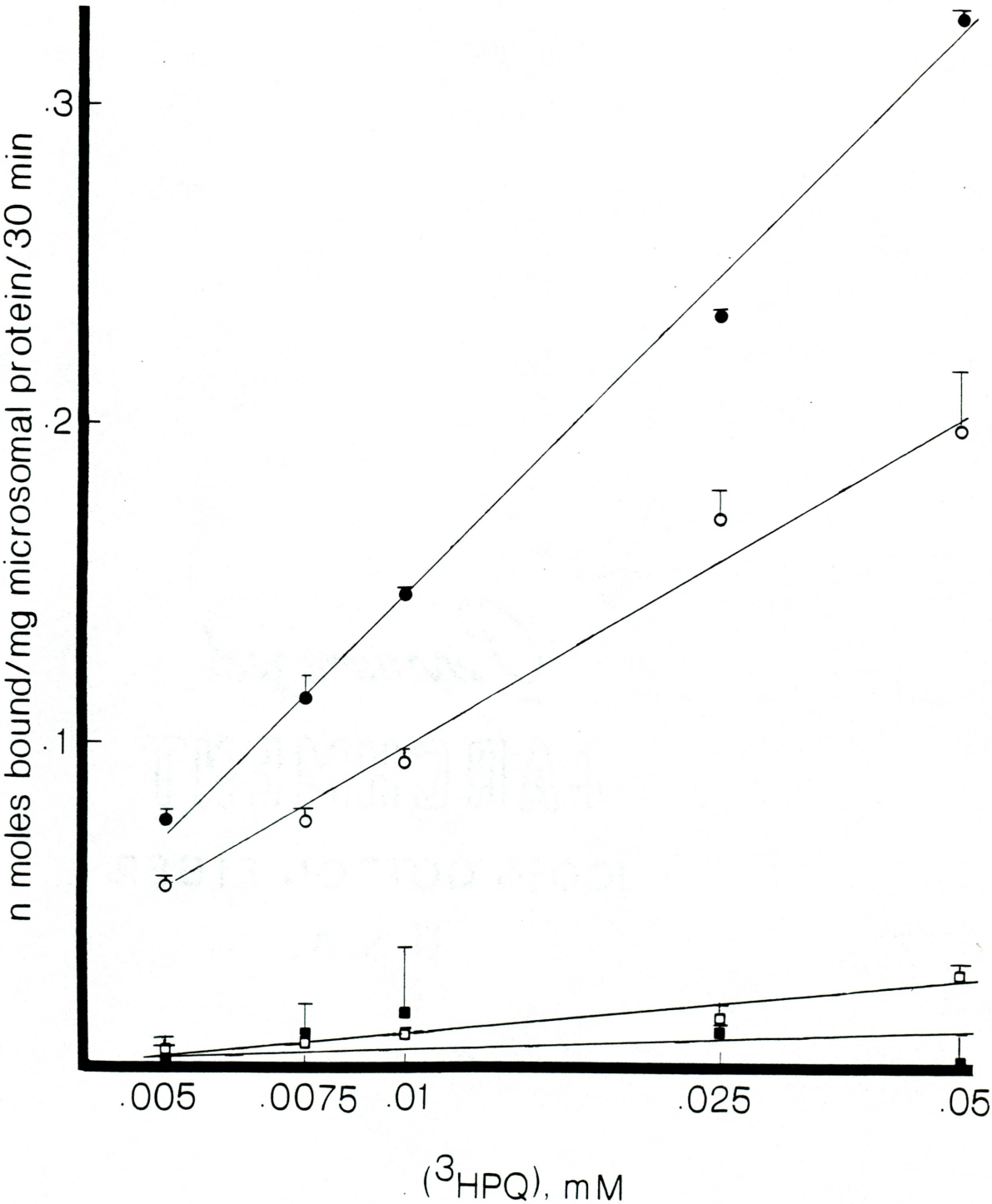
n moles bound/mg microsomal protein/30 min
.3
.2
.1
.005
.0075
.01
.025
.05
(^{3}HPQ), mM

Fig. 4

Figure 5. Lineweaver-Burk plot of covalent binding of 0.005 mM to 0.05 mM ^{3}H-primaquine metabolites to control and phenobarbital pretreated mouse liver microsomal protein in vitro. Each point is the mean and standard error of 3 to 5 experiments. ●, net binding to microsomes from control mice, Vmax = 1.1 x 10^{-2} nmoles/mg protein/minute. O, net binding to microsomes from phenobarbital pretreated mice, Vmax = 1.7 x 10^{-2} nmoles/mg protein/minute. Apparent Km = 2.6 x 10^{-5}M for both types of microsomes.

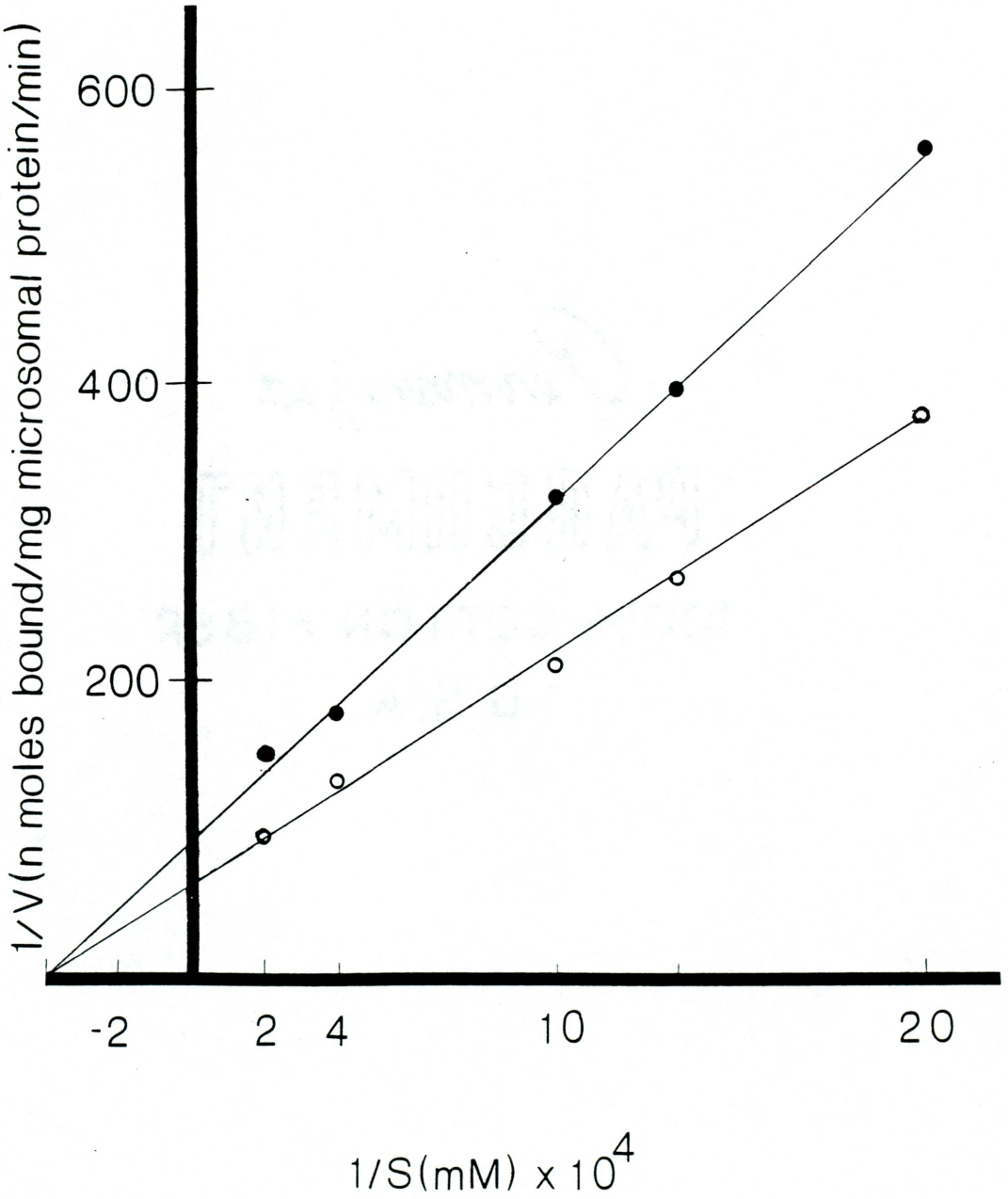
1/V(n moles bound/mg microsomal protein/min)
600
400
200
-2
2
4
10
20
1/S(mM) x 10^4

Fig. 5

Figure 6. Covalent binding of ^{3}H-primaquine (10 mg/Kg to 80 mg/Kg) and ^{14}C-primaquine (10 mg/Kg) to control and phenobarbital pretreated mouse liver microsomes *in vivo*. Control mice: cytochrome P450 content was 0.76 ± 0.004 nmoles/mg protein, ● ^{3}H-primaquine injected, n=3; ▲, ^{14}C-primaquine injected, n=4. Phenobarbital pretreated mice (as described in "Methods") cytochrome P450 content was 1.81 ± 0.05 nmoles/mg protein, Δ, ^{14}C-primaquine injected, n=2.

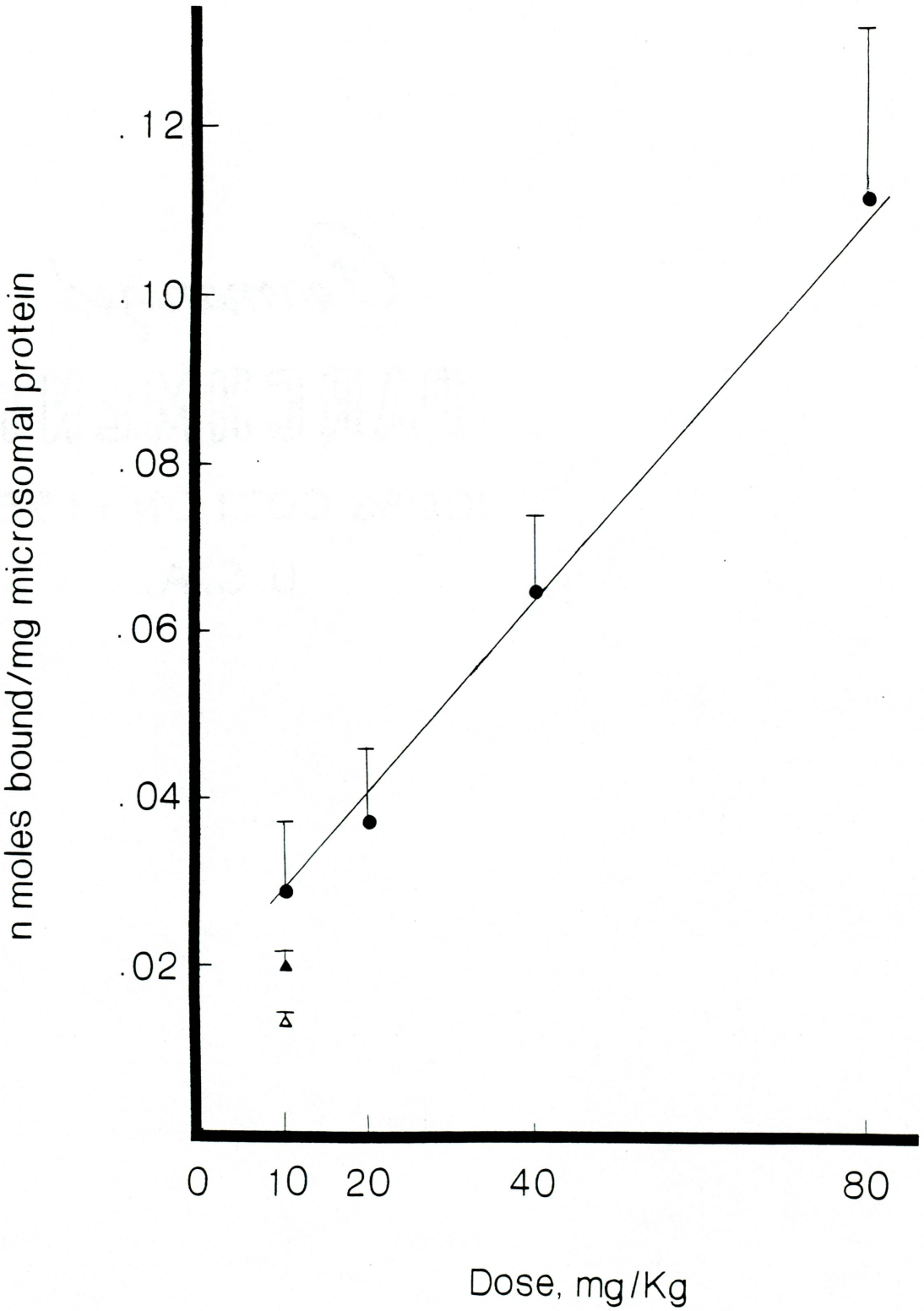
n moles bound/mg microsomal protein
.12
.10
.08
.06
.04
.02
0
10
20
40
80
Dose, mg/Kg

Fig. 6

Figure 7. Effect of 80 mg/Kg of primaquine on reduced glutathione (GSH) content of mouse liver *in vivo*. Each point is mean and standard error for 4 animals.

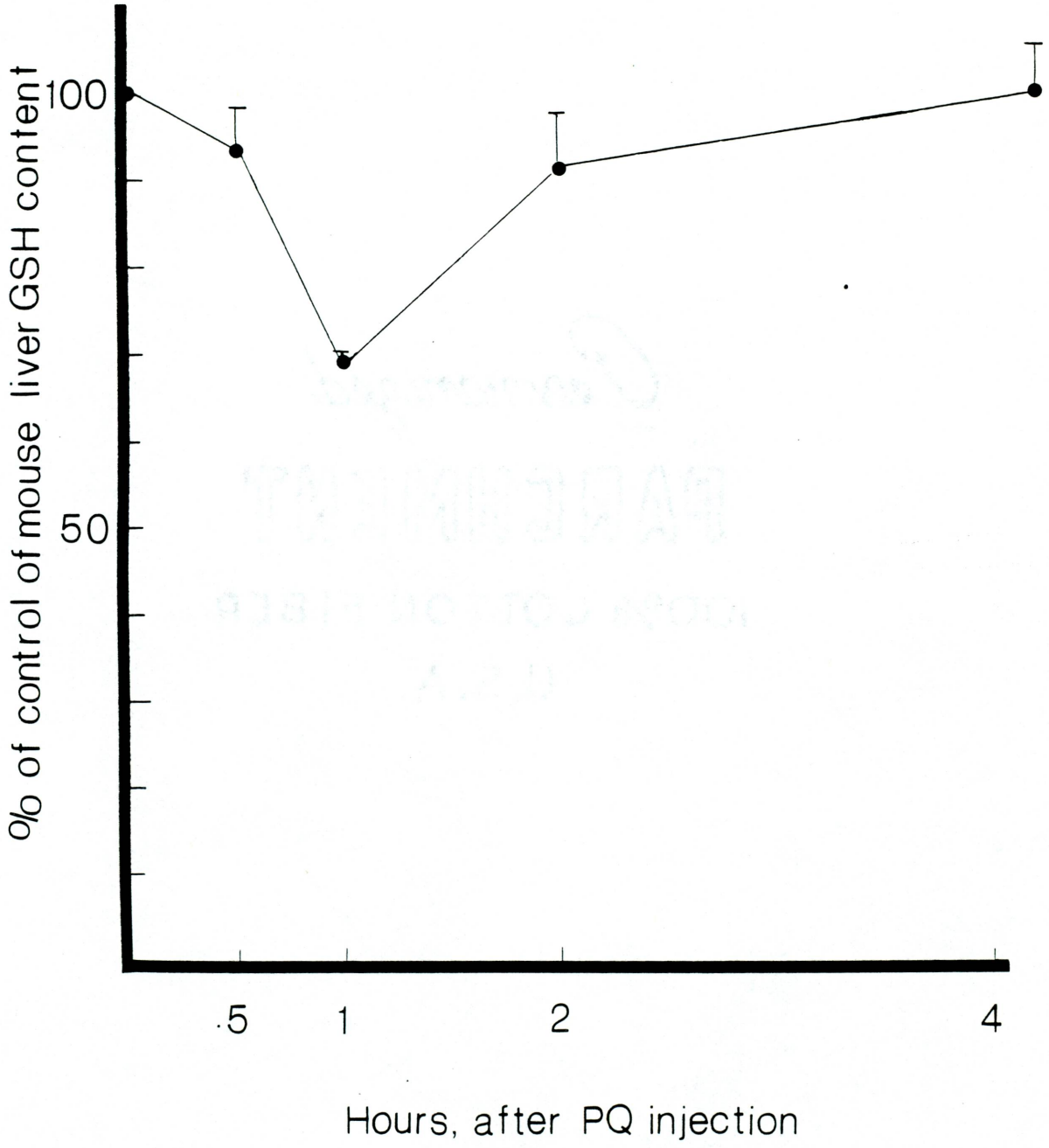
% of control of mouse liver GSH content
100
50
.5
1
2
4
Hours, after PQ injection

Fig. 7

Figure 8. Effect of 80 mg/Kg of primaquine on reduced glutathione (GSH) content of mouse erythrocytes *in vivo*. Each point is mean and standard error for 4 animals. ●——●, % control of GSH content.

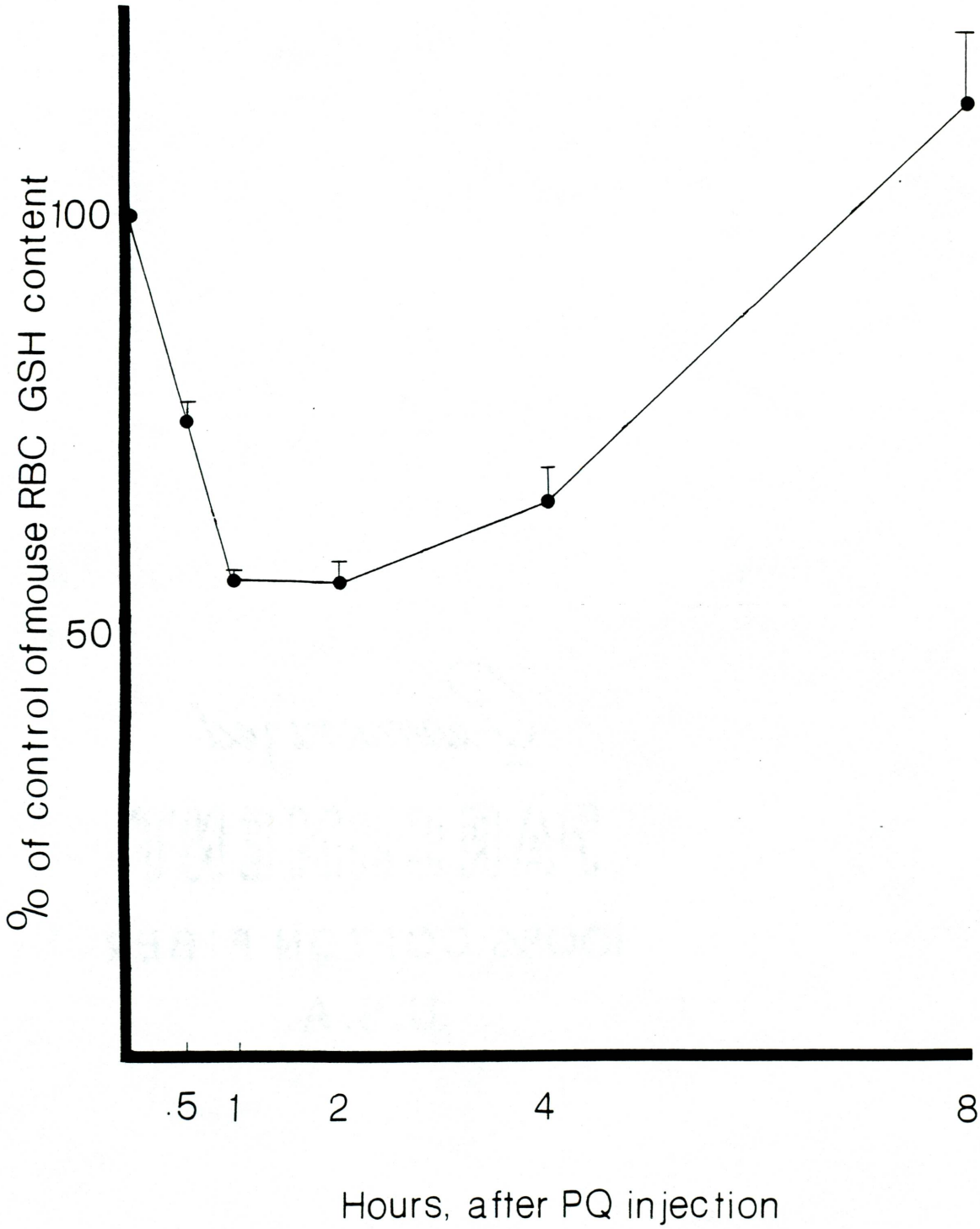
% of control of mouse RBC GSH content
100
50
.5
1
2
4
8
Hours, after PQ injection

Fig. 8

CHAPTER 3: COVALENT BINDING AND OTHER MECHANISMS OF PRIMAQUINE TOXICITY IN HUMAN BLOOD

Introduction

It has long been known that when primaquine (PQ, an antimalarial drug) is administered to sensitive men, an acute hemolytic anemia develops (Hockwald et al., 1952). However, the mechanism of this reaction is still not completely understood (Beutler, 1978).

Lipid peroxidation appears to play a significant role in drug-induced membrane damage. Liver microsomes undergo lipid peroxidative damage when they are incubated with NADPH. When erythrocytes were incubated with this peroxidative system, they hemolyzed (Rodgers et al., 1977). Many agents which deplete GSH in isolated hepatocytes also induce lipid peroxidation (Anundi et al., 1979). Doroshow et al. (1979) and Olson et al. (1980) reported that adriamycin decreased heart, liver and erythrocyte GSH levels and induced toxicity. Adriamycin treatment in rats inhibited microsomal cytochrome P450 and other enzymes activity; it also enhanced in vitro lipid peroxidation of liver microsomes by producing reactive oxygen species through its redox cycling and oxidized endogenous membrane α-tocopherol (Mimnaugh et al., 1981a, b). The protective roles of α-tocopherol, GSH, and other enzymatic defenses against radicals and oxidative damages are discussed by Fridovich (1978) and Chance et al. (1979).

Carbon tetrachloride is the best known compound which is converted to trichloromethyl radical by microsomal enzyme and induces toxicogenic events. This specie binds to protein and lipid covalently

(Reynolds, 1967) and also initiates peroxidation of membrane lipids (Recknagel and Glende, 1973). Other compounds which initiate toxic events through their radical metabolites are reviewed by Mason (1979) and Trush et al. (1982).

The chain reactions of membrane lipid peroxidation disrupt membrane structure integrity, and thus alter membrane-bound enzyme activities (van Boxten et al., 1979; Wills, 1971), membrane permeability (Hogberg et al., 1973), and finally increase membrane deformability and induce membrane breakages (Pasquali-Ronchetti et al., 1980).

Various drugs and foreign compounds have been found to be converted in the body to chemically reactive metabolites that bind covalently to tissue macromolecules thereby causing toxicities (Miller and Miller, 1966; Miller, 1970; Magee and Barnes, 1967; Judah et al., 1970; Mitchell et al., 1975). The magnitude of covalent binding parallels the severity of toxicity (Mitchell et al., 1973a, b; Jollow et al., 1973; Potter et al., 1973), and parallels the depletion of GSH content (Mitchell et al., 1973b).

A sharp depletion of the GSH of G6PD-deficient red cells followed by acute hemolysis occur after administration of PQ to susceptible subjects (Flanagan et al., 1958). This phenomenon emphasizes the importance of erythrocyte GSH for the maintenance of cell integrity (Fegler, 1952). Fraser and Vesell (1968a, b) found that hydroxylated metabolites of PQ, pentaquine, and acetanilid increased mechanical fragility and methemoglobin content but decreased GSH content in G6PD-deficient human erythrocytes more than that of normal

ones. Studies of Strother et al. (1981, 1983) also indicated that model metabolites of PQ, but not PQ itself, increased a dose-related methemoglobin formation in normal and G6PD-deficient erythrocytes. These metabolites depleted GSH content of G6PD-deficient red cells, whereas normal erythrocyte GSH levels were also depleted by high concentrations of PQ model metabolites.

The importance of membrane sulfhydryl groups for the integrity of the red blood cells and for the maintenance of cellular enzymes activity have been demonstrated by some investigators (Rapport and Scheuch, 1960; Scheuch et al., 1961). The destructibility of red cells might be due to the oxidation of their membrane sulfhydryl groups (Jacob and Jandl, 1962a, b). Szeinberg and Clejan (1964) reported that the protein sulfhydryl groups and GSH in whole blood cells of G6PD-deficient individuals were significantly lower than those of normal subjects. In contrast, there were no significant differences in the membrane sulfhydryl groups and GSH contents between the two kinds of red cells.

Previous work (Chau et al., 1983) has shown that PQ metabolite(s) depleted hepatic as well as erythrocyte GSH levels and were bound to mouse liver microsomal protein covalently. PQ has also been found to generate reactive oxygen species (Cohen and Hochstein, 1964; Summerfield and Tudhope, 1978; Fraser et al., 1975, 1981). It is possible that PQ toxicity is mediated by lipid peroxidation of red cell membranes through reactive oxygen species or by covalent binding of its reactive metabolite(s) to red cell membranes, hemoglobin and other

proteins after cellular antioxidative and other defensive mechanisms are diminished (Bus and Gibson, 1979; Chance et al., 1979).

In the investigation we are reporting here, we have measured the lipid peroxidation of mice liver microsomes and human erythrocytes as well as their membranes by PQ and its model metabolites. Covalent binding of PQ to human erythrocytes as well as their membranes and hemoglobin has been studied. Effects of α-tocopherol on lipid peroxidation, covalent binding and methemoglobin formation are reported. The effect of PQ and its derivatives on sulfhydryl groups of normal and G6PD-deficient erythrocyte membranes was also determined.

Materials and Methods

Chemicals

Primaquine diphosphate (PQ) was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. ^{3}H-PQ, labelled in the ring system, was purchased from New England Nuclear Corporation, Boston, MA, and recrystallized in the laboratory with unlabelled PQ to obtain a specific activity of 0.137 mCi/mmol. The purity was over 90% as determined by thin layer chromatography (TLC) with methanol-benzene (2:1) on silica gel (Strother et al., 1981). The 5-hydroxy-6-demethylprimaquine hydrobromide (5H6DPQ) was synthesized as described by Allahyari et al. (1983). The blue derivative of 5H6DPQ was prepared in the laboratory by extracting 5H6DPQ.3HBr in 1N NaOH with methylene chloride. The extract turned blue upon exposure to sunlight. The blue compound was purified by TLC and its identity verified by mass spectrophotometry as

described by Strother et al. (1983). Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate monosodium salt (G6P), glucose-6-phosphate-dehydrogenase (G6PD), glutathione (reduced form), bovine serum albumin, human hemoglobin (Type IV), α-tocopherol acid succinate, 2-thiobarbituric acid and 1,1,3,3-tetraethoxypropane were obtained from Sigma Chemical Company, St. Louis, MO. Magnesium chloride was purchased from J. T. Baker Chemical Company, Phillipsburg, NJ. Sephadex G-75 superfine was supplied by Pharmacia Fine Chemicals, Inc., Piscataway, NJ. CytoScint scintillation cocktail was obtained from WestChem, San Diego, CA. All other chemicals were of highest purity available.

Preparation of Microsomes

Male Swiss-Webster mice, obtained from Simonsen Laboratories, Gilroy, CA and weighing 22-27 g, were fed regular Purina Lab Chow and water ad libitum. They were killed by cervical dislocation, the livers were dissected out immediately and the gallbladder and other extraneous tissue rapidly removed. The livers were then blotted on absorbent paper, quickly weighed, and placed in ice-cold 1.15% KCl-0.025 M Tris buffer, pH 7.35. A 33% suspension of microsomes, in the same buffer, was prepared as described by Peters and Strother (1971).

Preparation of 50% Human Erythrocyte Suspension and Erythrocyte Membranes (Ghosts)

Blood specimens were obtained by venipuncture from normal and G6PD-deficient volunteers with disodium edetate as anticoagulant. The 50% washed erythrocyte suspension was prepared in 0.85% buffered

saline containing 10 mM dextrose as described by Fraser and Vesell (1968a). Human erythrocyte ghosts were prepared from washed, packed cells by the method described by Fairbanks et al. (1971).

Protein content was measured by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the reference standard. Hemoglobin concentration was determined by reading the absorbance (at 540 nm) in cyanmethemoglobin reagent (Hycel Inc., Houston, TX) using human hemoglobin (Sigma Chemical Co., Type IV) as the reference standard.

Lipid Peroxidation

Mouse liver microsomes (1.75 mg), human erythrocytes (0.5 ml of 50% RBC suspension), or human erythrocyte membranes (4.0 mg) were incubated alone or with PQ, 5H6DPQ, or the blue derivative of 5H6DPQ (at concentrations indicated in "Results") in the presence or absence of an NADPH-generating system (cofactors) in 0.5 ml 0.25 M Tris buffer (pH 7.35) containing NADP (3.3 μmol), G6P (35 μmol), $MgCl_2$ (15 μmol), and G6PD (2 units). The blue derivative of 5H6DPQ was dissolved in methanol, placed in the incubation flasks and the methanol was evaporated under a stream of N_2 prior to addition of any other materials. Tris buffer (0.25 M, pH 7.35) was added to bring the final volume of the incubation mixtures to 1.75 ml. Incubation was carried out at 37°C under air in a Dubnoff shaking incubator for varying times. Zero-time blanks containing all the components of the incubation mixtures were also included. In some experiments, α-tocopherol was added in a small volume of absolute alcohol to produce a final concentration of

0.01 mM. Lipid peroxidation was quantitated by spectrophotometric determination of the malondialdehyde-thiobarbituric acid complex (Bernheim et al., 1948). At the end of the incubation, 0.75 ml of cold 2.0 M TCA-1.7 M HCl was added to each incubation flask. The incubation mixtures were transferred to centrifuge tubes and centrifuged at 1000 x g for 10 minutes. To each 0.5 ml of the clear supernatant, 2.0 ml of 1.0% w/v 2-thiobarbituric acid reagent (Kohn and Liversedge, 1944) was added and incubated at 95°C for 15 minutes and cooled on ice. The absorbance was measured at 533 nm on a Beckman Acta MVI UV-visible spectrophotometer. Standard malondialdehyde was prepared by the acid hydrolysis of tetraethoxy-propane.

Binding to Mouse Liver Microsomal Protein

^{3}H-PQ was incubated with mouse liver microsomes, containing α-tocopherol in absolute ethanol or absolute ethanol, with or without an NADPH-generating system in 1.0 ml 0.25 M Tris buffer (pH 7.35) containing NADP (4 μmol), G6P (25 μmol), $MgCl_2$ (30 μmol) and G6PD (2 units). The incubation conditions and method of extraction of the mouse liver microsomal protein were previously described (Chau et al., 1983).

Binding to Human Erythrocytes

^{3}H-PQ was incubated with 1.0 ml 50% human erythrocytes suspension and mice liver microsomes (10 mg) in the presence or absence of an NADPH-generating system as described previously. After incubation at 37°C for 60 minutes under air in a Dubnoff shaking incubator, the incubation mixtures were centrifuged at 500 x g for 10 minutes. The

supernatant was aspirated from the erythrocytes as much as possible. The bottom-most 0.3 ml of the packed erythrocytes was transferred to 13 ml centrifuge tubes and lysed with 0.9 ml of distilled water. Absolute methanol (3.0 ml) was added to the lysed erythrocytes and the tubes were centrifuged again at 1000 x g for 10 minutes. The protein pellets were extracted 3 times with 2 ml hexane and then 9 times with 80% methanol as previously described (Chau et al., 1983). The extracted protein pellet was dissolved in 1.0 ml 10% sodium dodecyl sulfate and an aliquot of 20 µl was added to 10.0 ml CytoScint scintillation cocktail. Radioactivity was determined in a Beckman LS-250 scintillation counter.

Binding to Human Erythrocyte Membranes (Ghosts)

^{3}H-PQ was incubated with human erythrocyte membranes (4.0 mg) and mouse liver microsomes (10.0 mg) with or without an NADPH-generating system. The protein was precipitated by 10% TCA and extracted as described above.

Binding to Human Hemoglobin

A column (35 x 1.4 cm) of superfine Sephadex G-75 was packed in equilibrium with 0.05 M phosphate buffer, pH 7.8, containing 0.15 M NaCl. Human hemoglobin, Type IV, 100 mg/ml in the same buffer was reduced by excess sodium dithionite, added to the column and eluted with the same buffer. The main band was collected and stored under N_2 (Summerfield and Tudhope, 1978). ^{3}H-PQ was incubated for 60 minutes with the eluted hemoglobin (75 mg) and mouse liver microsomes (10 mg) in the presence or absence of an NADPH-generating system as described

above. The incubation mixtures were transferred to ultracentrifuge tubes at the end of the incubation. The tubes were centrifuged at 105,000 x g for 30 minutes in a Beckman Model L3-50 ultracentrifuge. The microsome free supernatant was placed on the Sephadex G-75 column and eluted with 0.05 M pH 7.8 phosphate buffer containing 0.15 M NaCl. The effluent was monitored at 280 nm and fractions were collected. An aliquot of 100 µl of each fraction was added to 10 ml CytoScint cocktail and counted for radioactivity.

Methemoglobin Formation and Assay

PQ, 5H6DPQ and the blue derivative of 5H6DPQ were incubated with 0.5 ml of a 50% normal or G6PD-deficient erythrocyte suspension at 37°C for 60 minutes in a Dubnoff shaking incubator. Sufficient Tris buffer (0.25 M, pH 7.35) was added to the incubation mixtures to bring the final volume to 1.75 ml. The flasks were placed in ice for 5 minutes at the end of the incubation and the contents then transferred to centrifuge tubes. The tubes were centrifuged at 1000 x g for 10 minutes. The supernatant was aspirated and the packed erythrocytes were washed twice with 2.0 ml 0.85% buffered saline containing 10 mM dextrose. An aliquot of 0.1 ml of the resuspended 50% erythrocytes suspension in the above buffer was used for methemoglobin assay as described by Fraser and Vesell (1968a). In some experiments α-tocopherol in absolute ethanol was added to the incubation mixture to study its effects on methemoglobin formation by 5H6DPQ.

Mice were injected with PQ (80 mg/Kg, i.p.) and sacrificed by cervical dislocation at various times after PQ administration. Blood

was collected by cardiac puncture with heparin as anticoagulant. The blood was centrifuged at 1000 x g for 10 minutes. The packed erythrocytes were washed with 0.85% buffered saline containing 10 mM dextrose and methemoglobin assays were performed as described above.

Effects of Primaquine and Its Metabolites on Normal and G6PD-Deficient Erythrocyte Membrane Sulfhydryl Levels

PQ, 5H6DPQ, or the blue derivative of 5H6DPQ was incubated with 4.0 ml of normal or G6PD-deficient red blood cells (50% suspension). A sufficient volume of 0.25 M Tris buffer, pH 7.35 was added to bring the final volume of incubation mixtures to 5.0 ml. Incubation was carried out at 37°C under air for 60 minutes in a Dubnoff shaking incubator. Incubation flasks were kept in ice for 5 minutes at the end of the incubation. The incubation mixtures were then transferred to centrifuge tubes and centrifuged at 1000 x g for 10 minutes. The supernatant was aspirated and the packed erythrocytes were lysed with 40 ml of cold 4 mM Na phosphate buffer, pH 8.0, and ghosts were prepared by the method of Fairbanks et al. (1971). The ghosts were resuspended in 0.5 ml of 0.02 M disodium EDTA and a sufficient volume of the same sodium phosphate buffer up to the original volume of the packed cells. An aliquot of the ghost suspension (0.4 ml) was mixed with 4.55 ml of 0.01 M sodium phosphate buffer (pH 8.0) and 0.05 ml of 0.01 M DTNB in absolute methanol. The solution was kept in the dark at room temperature for 50 minutes and then centrifuged at 17,000 x g for 5 minutes (Szeinberg and Clejan, 1964). The absorbance at 412 nm was measured against both a reagent blank (no tissue) and a tissue blank (no DTNB).

Statistics

Data were expressed as the mean ± S.E.M. The significance of the difference between means was determined by Student's t-test. Statistical differences were considered to be significant when $p < 0.05$.

Results

Lipid Peroxidation and Covalent Binding in Microsomes

As expected, Table 10 shows that lipid peroxidation in mouse liver microsomes was markedly enhanced by the presence of the cofactor mixture which generated NADPH. When 0.1 mM PQ was added there was a marked decrease in lipid peroxidation indicating substrate inhibition of NADPH-induced microsomal lipid peroxidation as has been reported for other compounds (Miles et al., 1980). The time course of lipid peroxidation over 60 minutes in the presence of 0.1 mM PQ, 5H6DPQ or the blue derivative of 5H6DPQ was examined with the results shown in Figure 9; zero time values were subtracted from those at all other incubation intervals. Most of the lipid peroxidation occurred in the first 15 minutes; following this period the results were quite variable except for a steady increase in lipid peroxidation with 5H6DPQ in the presence of cofactors. Overall, 5H6DPQ was the most active in producing lipid peroxidation while its blue derivative was the least active; in all cases the effect was greater in the presence of NADPH generated by the cofactor mixture.

Addition of α-tocopherol (0.01 mM) produced more than a 50% inhibition of lipid peroxidation in the presence of primaquine and

cofactors as shown in Table 11. The results of a comparable study of the effect of α-tocopherol on the covalent binding of tritiated PQ to mouse liver microsomal protein are shown in Table 12. There was a 30% inhibition of covalent binding when α-tocopherol in absolute alcohol was added to the incubation mixture in the binding studies, whereas the inhibition effect contributed by absolute ethanol alone was only 10%. It appears that α-tocopherol inhibited lipid peroxidation in the presence of PQ significantly more than it inhibited the covalent binding of PQ.

Lipid Peroxidation in Erythrocytes

As shown in Table 12, considerable lipid peroxidation occurred in intact erythrocytes incubated for 60 minutes; addition of the cofactor mixture produced a small increase in the amount of peroxidation. When PQ was added in the absence of cofactors there was much less lipid peroxidation than in the control; addition of cofactors removed this inhibitory effect of PQ so that peroxidation was now a little greater than in the control with cofactors. Quite similar results were obtained with 5H6DPQ and the blue derivative from 5H6DPQ, although they were less inhibitory to lipid peroxidation in the absence of cofactors.

The results of a study of the time course of lipid peroxidation in human erythrocyte membranes in the presence of 5H6DPQ and with and without cofactors are shown in Figure 10. 5H6DPQ significantly inhibited the NADPH-induced lipid peroxidation in erythrocyte membranes in contrast to the lack of such inhibition in intact erythrocytes.

Methemoglobin (metHb) Formation in Erythrocytes

As reported previously (Strother et al., 1981, 1983), low (0.1-0.005 mM) concentrations of 5H6DPQ readily oxidize Hb to metHb in both normal and G6PD-deficient erythrocytes. A comparison of metHb formation by 0.1 mM PQ, 5H6DPQ and the blue derivative of 5H6DPQ in normal and G6PD-deficient human erythrocytes after a sixty minute incubation is shown in Table 14. 5H6DPQ was much more active than PQ or the blue derivative and produced significantly more metHb in G6PD-deficient than in normal erythrocytes. In contrast to its effect on lipid peroxidation and covalent binding, α-tocopherol did not inhibit methemoglobin formation by 5H6DPQ in normal erythrocytes (Table 15).

Covalent Binding in Erythrocytes

In Table 16 are shown the results of studies on the covalent binding of 0.01 mM ^{3}H-PQ to total protein of both normal and G6PD-deficient erythrocytes incubated with mouse liver microsomes with and without the cofactor mixture present. There was a significant net binding attributable to the presence of the cofactors. Also the net binding to the protein of G6PD-deficient cells was significantly greater than that to normal erythrocytes.

The possibility that PQ or its metabolites may bind covalently to the proteins of erythrocyte membranes was examined by incubating 0.01 or 0.1 mM ^{3}H-PQ with mouse hepatic microsomes and human erythrocyte membranes in the presence or absence of the cofactor mixture. It was not possible to separate the microsomes from the erythrocyte membranes after the incubation so that it was only possible to measure

the total covalent binding to the combined microsomal and membrane protein. At both concentrations of PQ the net binding to the combined microsomal-membrane protein (attributable to the presence of cofactors) was significantly greater than that when PQ was incubated with mouse liver microsomes alone (Table 17). These results suggest that there was small but significant amount of primaquine metabolite(s) bound to human erythrocyte membrane proteins.

Since covalent binding to membranes could involve -SH groups, the effect of 0.1 mM PQ, 5H6DPQ or the blue derivative of 5H6DPQ on the -SH groups of normal and G6PD-deficient erythrocyte membranes was measured. Table 18 shows that all three compounds significantly decreased the amount of measurable sulfhydryl groups of G6PD-deficient human erythrocyte membranes but had no effect on normal erythrocyte membranes. The effect of 5H6DPQ was more marked than that of PQ or the blue derivative of 5H6DPQ.

The possibility that PQ or its metabolites may bind covalently to hemoglobin was examined by incubating human hemoglobin (Type IV) with ^{3}H-PQ and mouse liver microsomes as described under "Methods." As shown in Table 19 and Figures 11 and 12, after separation on a Sephadex G-75-40 column, a significant amount of radioactivity was found in the fractions from the incubation mixture containing cofactors which was significantly greater than that from the incubation mixture lacking cofactors. The radioactive peaks B and C of Figures 3 and 4 appear to be due to ^{3}H-PQ and breakdown products or metabolites of PQ; they were also observed when ^{3}H-PQ was chromatographed in 0.25 M Tris buffer on

the Sephadex G-75-40 column without prior incubation. Also the small absorbance peak at 280 nm from fractions 35 to 47 in Figure 12 may be attributable to primaquine metabolite(s) since model metabolites of primaquine did show some absorbance at that wavelength.

Oxidation of Hemoglobin in vivo

Table 20 shows that there is no significant amount of methemoglobin produced in vivo after injection of 80 mg/Kg of PQ.

Discussion

Although PQ has been found to produce reactive oxygen species (Cohen and Hochstein, 1964; Summerfield and Tudhope, 1978; Fraser et al., 1981) and the 5,6-dihydroxy derivative (5H6DPQ) may react with oxygen to produce hydrogen peroxide (Fraser et al., 1981) they did not stimulate NADPH-dependent microsomal lipid peroxidation in this study. In fact, PQ, 5H6DPQ, and the blue derivative from 5H6DPQ inhibited NADPH-induced microsomal lipid peroxidation markedly.

Many investigators have reported substrate inhibition of NADPH-induced microsomal lipid peroxidation (Orrenius et al., 1964; Pederson and Aust, 1974; Miles, 1980) but suggested different mechanisms. Orrenius et al. (1964) proposed that the inhibition is due to a competition between drug metabolism and lipid peroxidation for reducing equivalents derived from NADPH. Pederson and Aust (1974) concluded that the inhibition of lipid peroxidation is caused by the antioxidant properties of a drug metabolite which is formed in the presence of

NADPH. Miles et al. (1980) believe that inhibition of hepatic microsomal lipid peroxidation is independent of drug metabolism but is caused by the antioxidant properties of the substrates. PQ is known to be metabolized rapidly in the body (Tarlov et al., 1962) and has oxidant and redox properties (Flanagan et al., 1958; Tarlov et al., 1962). It seems likely that the inhibitory effect of PQ and its derivatives on hepatic microsomal lipid peroxidation is due to the competition between drug metabolism and lipid peroxidation for NADPH-reducing equivalents.

On the other hand, Anundi et al. (1979) and Smith et al. (1983) found that bromobenzene also shows some degree of substrate inhibition of lipid peroxidation and they suggested that the lipid peroxidation which occurs during bromobenzene toxicity is due to the depletion of GSH and is a consequence of cell death.

The small amount of NADPH-induced lipid peroxidation in the presence of PQ is reduced by α-tocopherol to less than 50%. α-Tocopherol is a well known antioxidant and radical scavenger. It interrupts the free radical chain reactions of lipid peroxidation (Bus and Gibson, 1979; Chance et al., 1979).

The inhibition of covalent binding of PQ metabolite(s) to mouse liver microsomal protein by α-tocopherol is probably due to its antioxidant effect which spared hepatic GSH from oxidation and thus protect tissue macromolecules against electrophilic attack by PQ reactive metabolite(s).

Substrate inhibition of NADPH-dependent lipid peroxidation was

also found in human erythrocytes and erythrocyte membranes in the presence of PQ, 5H6DPQ and the blue derivative of 5H6DPQ. In order to ensure the presence of sufficient reducing equivalents, an NADPH-generating system was added to human intact erythrocytes and erythrocytes membranes in lipid peroxidation studies. The addition of cofactors to intact red cells (Table 13) may enhance the redox cycling reaction between oxyhemoglobin and PQ as well as its derivatives, increase reactive oxygen species production, and thus potentiate lipid peroxidation.

5H6DPQ is more active in producing methemoglobin in vitro than PQ and the blue derivative from 5H6DPQ, especially in G6PD-deficient red cells as reported by Strother et al. (1981, 1983). This result indicates that a PQ metabolite(s) is more potent than PQ itself in inducing toxicity. G6PD-deficient cells are more vulnerable than normal cells to oxidative damage. Even though PQ can deplete hepatic and erythrocyte GSH contents as reported previously (Chau et al., 1983) it does not produce a significant amount of methemoglobin in vivo. In contrast to its effect on lipid peroxidation and covalent binding, α-tocopherol does not inhibit in vitro methemoglobin formation by 5H6DPQ in normal red cells.

The fact that PQ metabolite(s) decreased sulfhydryl groups of G6PD-deficient human erythrocyte membranes significantly but had no effect on those of normal red cell membranes suggests that covalent binding may be one of the mechanism of PQ-induced hemolysis. The results of other studies in our laboratory (unpublished data) with

5H6DPQ and its blue derivative, show a dose-related decrease of GSH contents of G6PD-deficient erythrocytes but not of normal cells. Flanagal et al. (1958) have also found that an acute destruction of GSH precedes hemolysis in PQ-sensitive subjects. Further evidence is provided by the fact that the PQ metabolite(s) binds to human erythrocyte total protein, hemoglobin and plasma membrane protein covalently. G6PD-deficient erythrocytes are more prone to covalent binding. Furthermore, Beutler et al. (1957) and Tarlov et al (1962) did not find an equivalent rise in oxidized glutathione (GSSG) to the fall in GSH. Beutler et al. (1957) suggested that GSSG may change to hydrogen sulfide eventually, and Tarlov et al. (1962) explained that there may be a formation of mixed sulfides of GSH with sulfhydryl groups of the globin molecule. We suggest that the decrease of GSH may be due to covalent binding in addition to oxidation.

A recent study of Kelman et al. (1982) indicated that PQ lowers cellular NADPH level but not GSH content, and stimulate the hexose monophosphate shunt in normal human erythrocytes. These investigators agree with Cohen and Hochstein (1964) that PQ mediates H_2O_2 formation in oxyhemoglobin-containing red cells and PQ reacts with NADPH to produce H_2O_2.

In normal red cells, the activity of G6PD is increased by the decreased NADPH levels, which is either lowered by direct interaction with PQ (Kelman et al., 1982) or by consumption in glutathione reductase reactions to reduce GSSG or peroxides (Cohen and Hochstein, 1964), or by the combination of both reactions, and thus glucose-6-

phosphate is oxidized at a higher rate to regenerate an adequate amount of NADPH. In G6PD-deficient erythrocytes, as the hexose monophosphate pathway activity is diminished, adequate NADPH levels cannot be maintained under oxidative stress.

The result of this study and others suggest the following sequence of events leading to PQ induced hemolysis: (1) Cellular GHS content is depleted by PQ metabolite(s) and reactive oxygen species produced from PQ through covalent binding, conjugation, or oxidation. (2) The G6PD-deficient erythrocytes are incapable of maintaining an adequate amount of NADPH for the reduction of GSSG and mixed disulfide of GSH and protein to regenerate GSH by way of the glutathione reductase reaction. GSH is important in maintaining the sulfhydryl groups of red cell enzymes and membranes in the reduced state (Beutler, 1978), and in removing the harmful hydrogen peroxide and organic peroxides in erythrocytes. The GSH depletion renders the red cell membranes susceptible to oxidative change and red cell enzymes to inactivation. (3) PQ metabolite(s) oxidize(s) and binds to vital red cell membrane proteins, hemoglobin, and enzymes after the protective role of GSH is decreased. (4) Red cell membrane permeability is then changed (Weed, 1961), enzyme functions are altered or destroyed and eventually the lysis of erythrocytes occurs (Beutler, 1954).

Table 10. Effect of 0.1 mM primaquine (PQ) and cofactors (CM) on lipid peroxidation in mouse hepatic microsomes

	nmoles malondialdehyde equivalent/mg microsomal protein[a]		
Condition	0 minutes	60 minutes	60 min-0 min.
Microsome	0.78 ± 0.20	7.00 ± 0.79	6.22 ± 0.65
Microsome + CM	0.85 ± 0.03	52.17 ± 6.50	51.33 ± 6.50[b]
Microsome + PQ	0.79 ± 0.08	2.41 ± 0.19	1.62 ± 0.35
Microsome + PQ + CM	0.79 ± 0.06	2.88 ± 0.33	2.11 ± 0.35[c]

[a]Values shown are means and standard errors for 3 to 7 experiments.

[b]Significantly different from microsome incubated without CM at $p < 0.001$

[c]Significantly different from microsome incubated with CM at $p < 0.001$

Table 11. Effect of 0.01 mM α-tocopherol on lipid peroxidation in mouse liver microsomes by 0.01 mM primaquine (PQ) in the presence or absence of cofactors (CM)

	nmoles malondialdehyde equivalent/mg microsomal protein[a]	
Drug	No Cofactors	With Cofactors
PQ (control)	0.94 ± 0.26	8.06 ± 0.37
PQ + α-tocopherol in absolute ethanol	0.53 ± 0.17	3.44 ± 0.65[b]
PQ + absolute ethanol	1.18 ± 0.35	9.80 ± 0.48[c]

[a]Values shown are means and standard errors for 4 experiments. Zero time values was subtracted from values at 60 minutes of incubation.

[b]Significantly different from control with CM at $p < 0.005$

[c]Significantly different from control with CM at $p < 0.025$

Table 12. Effect of 0.01 mM α-tocopherol on covalent binding of 0.01 mM ^{3}H-primaquine (^{3}H-PQ) to mouse liver microsomal protein *in vitro* in the presence or absence of cofactors

Drug	nmoles bound/mg protein/60 minutes[a]			
	No Cofactors	With Cofactors	Net Bound	% Inhibition
^{3}H-PQ (control)	0.05 ± 0.01	0.26 ± 0.001	0.21 ± 0.01	
^{3}H-PQ + α-tocopherol in absolute ethanol	0.04 ± 0.004	0.19 ± 0.01	0.15 ± 0.01[b]	30%
^{3}H-PQ + absolute ethanol	0.04 ± 0.004	0.24 ± 0.004	0.19 ± 0.003[c]	10%

[a]Values shown are means and standard errors for 3 to 4 experiments.

[b]Significantly different from control at $p < 0.005$

[c]Significantly different from control at $p < 0.025$

Table 13. Lipid peroxidation of human erythrocytes by primaquine and its model metabolites

Drug	nmoles malondialdehyde equivalent/gm Hb[a]	
	No Cofactors	With Cofactors
None (control)	50.8 ± 17.4	74.0 ± 11.5
0.1 mM PQ	21.9 ± 5.0	84.1 ± 31.2
0.1 mM 5H6DPQ	39.7 ± 15.6	79.4 ± 27.0
0.1 mM blue compound from 5H6DPQ	36.5 ± 9.3	91.4 ± 8.3

[a]Values shown are means and standard errors for 4 experiments. Zero time values were subtracted from values at 60 minutes of incubation.

Table 14. Effect of 0.1 mM primaquine, 5H6DPQ and blue compound from 5H6DPQ on methemoglobin formation of normal and G6PD-deficient erythrocytes *in vitro*

	Percent methemoglobin[a]	
Drug	Normal RBC	G6PD deficient RBC
None (control)	3.0 ± 0.9	2.1 ± 0.7
PQ	1.7 ± 0.4	3.4 ± 0.6[b]
5H6DPQ	14.2 ± 0.7	24.8 ± 2.0[c]
Blue compound from 5H6DPQ	5.7 ± 0.8	5.6 ± 0.8

[a]Values shown are means and standard errors for 4 experiments.

[b]Significantly different from normal RBC at $p < 0.05$

[c]Significantly different from normal RBC at $p < 0.005$

Table 15. Effect of 0.01 mM α-tocopherol on methemoglobin formation by 0.1 mM 5H6DPQ *in vitro*

Drug	Percent methemoglobin[a]
None (control)	2.2 ± 0.4
5H6DPQ	12.4 ± 0.8[b]
5H6DPQ + α-tocopherol in absolute ethanol	13.5 ± 0.6[b]
5H6DPQ + absolute ethanol	12.0 ± 1.0[b]

[a]Values shown are means and standard errors for 4 experiments.

[b]Significantly different from control at $p < 0.001$

Table 16. Covalent binding of 0.01 mM ^{3}H-primaquine to normal and G6PD-deficient human erythrocyte protein *in vitro*

	pmoles bound/mg human RBC protein[a]		
RBC	No Cofactors	With Cofactors	Net Bound
Normal	7.1 ± 2.2	12.3 ± 2.3	5.2 ± 0.5
G6PD-deficient	8.6 ± 0.7	17.6 ± 2.6	9.0 ± 1.9[b]

[a]Values shown are means and standard errors for 3 to 4 experiments.

[b]Significantly different from normal RBC at $p < 0.05$

Table 17. Covalent binding of ^{3}H-Primaquine to mouse liver microsomal protein and human erythrocyte membrane *in vitro*

		nmoles bound/mg microsomal protein/4 mg RBC membrane[a]		
Condition	^{3}H-PQ Conc.	No Cofactors	With Cofactors	Net Bound
Microsome (control)	0.1 mM	0.50 ± 0.03	1.07 ± 0.06	0.57 ± 0.03
Microsome + RBC membrane	0.1 mM	0.46 ± 0.02	1.15 ± 0.02	0.69 ± 0.04[b]
Microsome (control)	0.01 mM	0.05 ± 0.01	0.20 ± 0.001	0.16 ± 0.01
Microsome + RBC membrane	0.01 mM	0.05 ± 0.003	0.25 ± 0.01	0.20 ± 0.02[c]

[a]Values shown are means and standard errors for 3 to 4 experiments.

[b]Significantly different from control (0.1 mM) at $p < 0.05$

[c]Significantly different from control (0.01 mM) at $p < 0.05$

Table 18. Effect of 0.1 mM primaquine, 5H6DPQ, and blue compound from 5H6DPQ on sulfhydryl groups of normal and G6PD-deficient human erythrocyte membrane *in vitro*

	-SH groups, mg/gm human RBC membrane protein[a]		
Drug	Normal RBC Membrane	G6PD-deficient RBC membrane	% Normal
None (control)	13.3 ± 1.0	9.7 ± 1.6	75%
PQ	12.1 ± 0.9	8.3 ± 1.5[b]	69%
5H6DPQ	12.8 ± 1.2	6.9 ± 0.9[c]	54%
Blue compound from 5H6DPQ	12.2 ± 0.8	8.1 ± 1.6[d]	66%

[a]Values shown are means and standard errors for 4 to 5 experiments.

[b]Significantly different from normal at $p < 0.05$

[c]Significantly different from normal at $p < 0.001$

[d]Significantly different from normal at $p < 0.05$

Table 19. Covalent binding of 0.01 mM ^{3}H-primaquine to human hemoglobin (Type IV) *in vitro*

nmoles bound/mg hemoglobin/30 minutes[a]		
No Cofactors	With Cofactors	Net Bound
0.038 ± 0.006	0.062 ± 0.003[b]	0.024 ± 0.003

[a]Values shown are means and standard errors for 3 experiments.

[b]Significantly different from binding without cofactors at $p < 0.025$

Table 20. Effect of 80 mg/Kg of primaquine on methemoglobin formation *in vivo*

Hours after PQ injection	Percent methemoglobin[a]
0	1.6 ± 0.5
0.5	3.6 ± 0.1
1	0.9 ± 0.3
2	2.2 ± 0.5
4	1.1 ± 0.1
8	2.7 ± 0.1

[a]Values shown are the mean and standard error for 4 animals.

Figure 9. Time course of effect of 0.1 mM primaquine, 5H6DPQ, and blue compound from 5H6DPQ on lipid peroxidation of mouse liver microsomes *in vitro*. Each point is mean and standard error for 3 to 6 experiments. Δ-------Δ, primaquine; ▲-------▲, primaquine + CM; □-------□ , 5H6DPQ; ■--------■ , 5H6DPQ + CM; o-------o, blue compound from 5H6DPQ; ●-------●, blue compound from 5H6DPQ + CM.

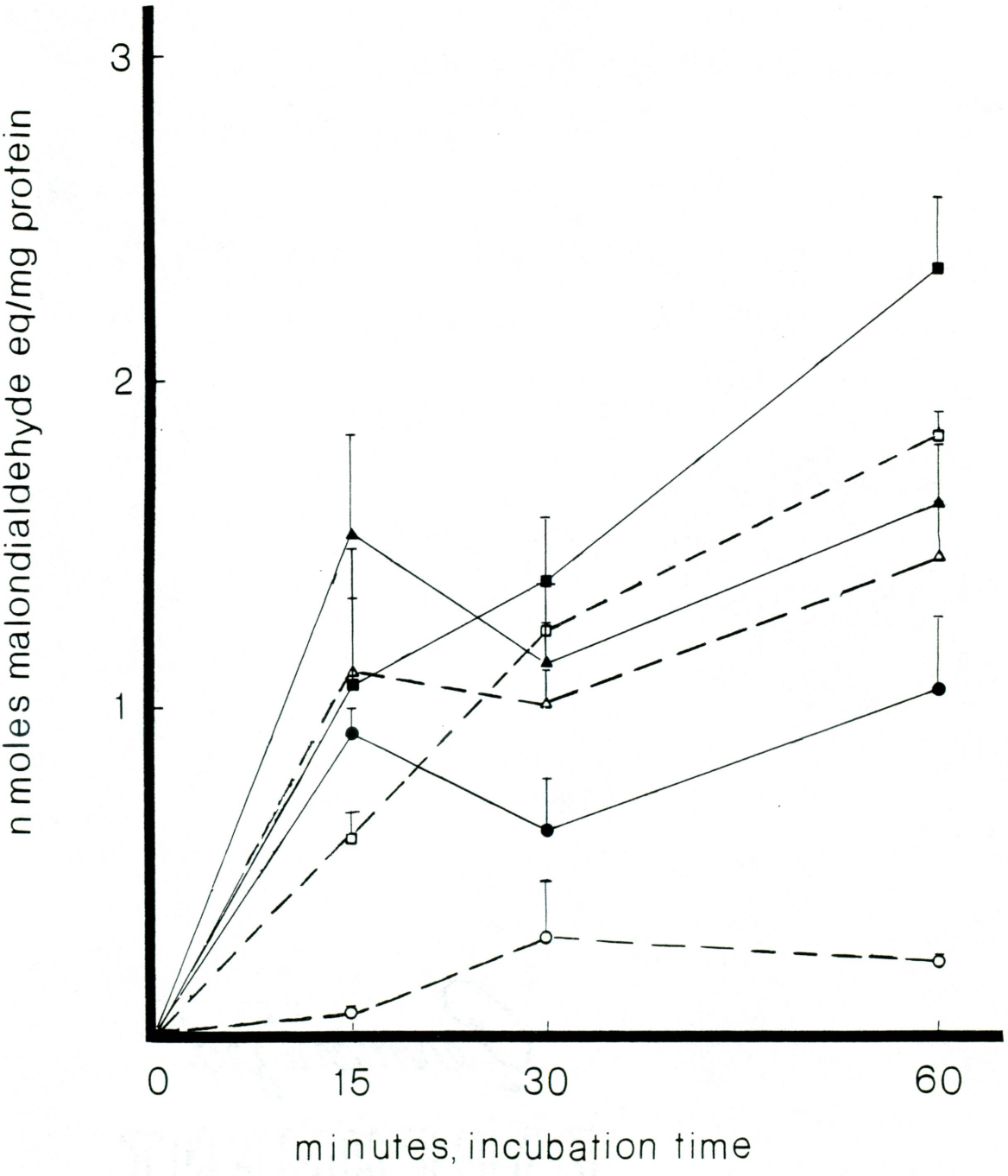
n moles malondialdehyde eq/mg protein
3
2
1
0
15
30
60
minutes, incubation time

Fig. 9

Figure 10. Time course of lipid peroxidation of human erythrocyte membrane. Each point is mean and standard error of 3 experiments. O-------O, RBC membrane; ●——●, RBC membrane + CM; □-------□, RBC membrane + 0.1 mM 5H6DPQ; ■——■, RBC membrane + 0.1 mM 5H6DPQ + CM. *Significantly different from RBC membrane + CM at $p < 0.025$.

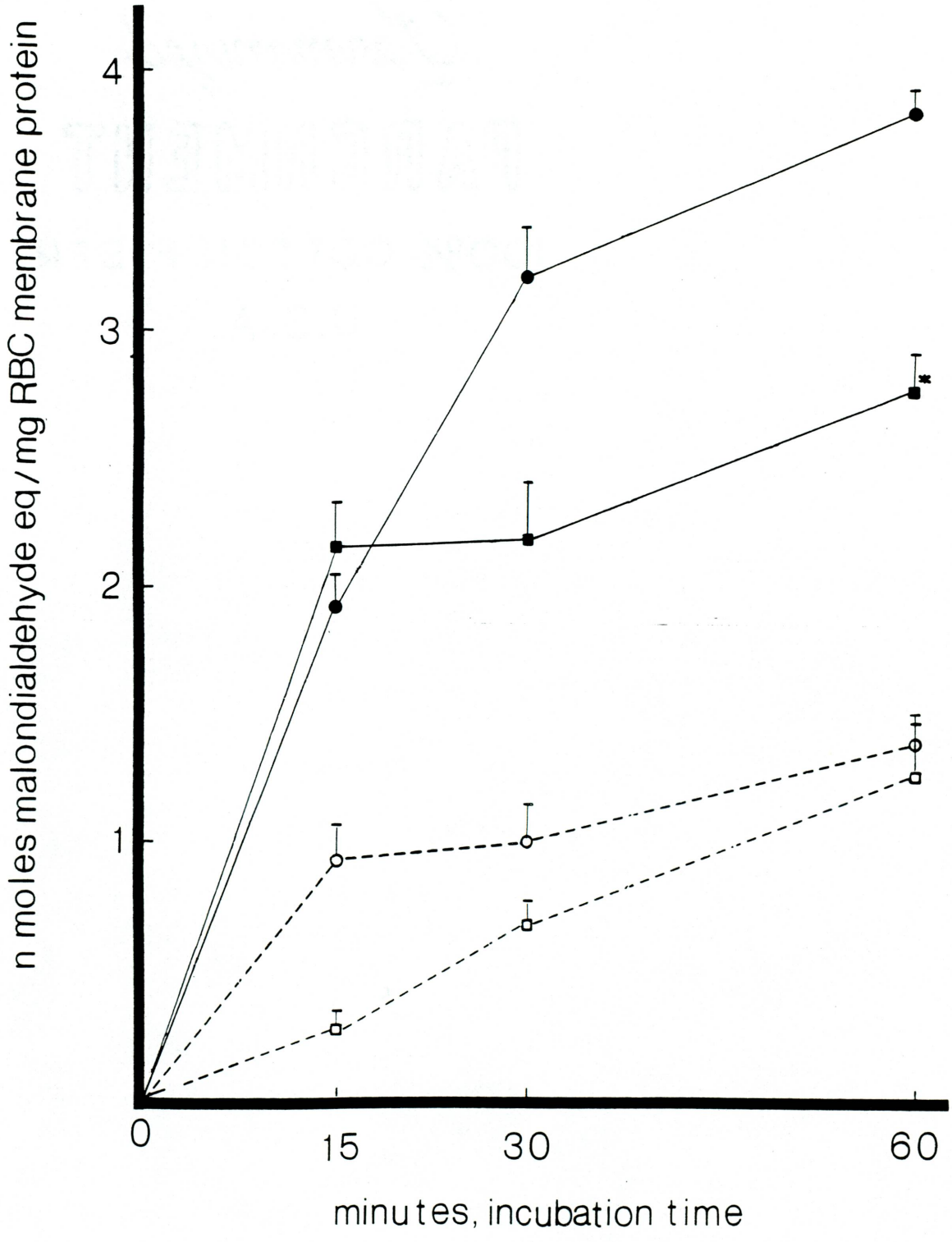
n moles malondialdehyde eq/mg RBC membrane protein
4
3
2
1
0
15
30
60
minutes, incubation time
*

Fig. 10

Figure 11. Column chromatography of human hemoglobin (Type IV) on Sephadex G-75-40 gel after incubated with 0.01 mM ^{3}H-primaquine and mouse liver microsomes *in vitro*. All fractions were counted for radioactivity. --------, A_{280}; ●——●, radioactivity in dpm.

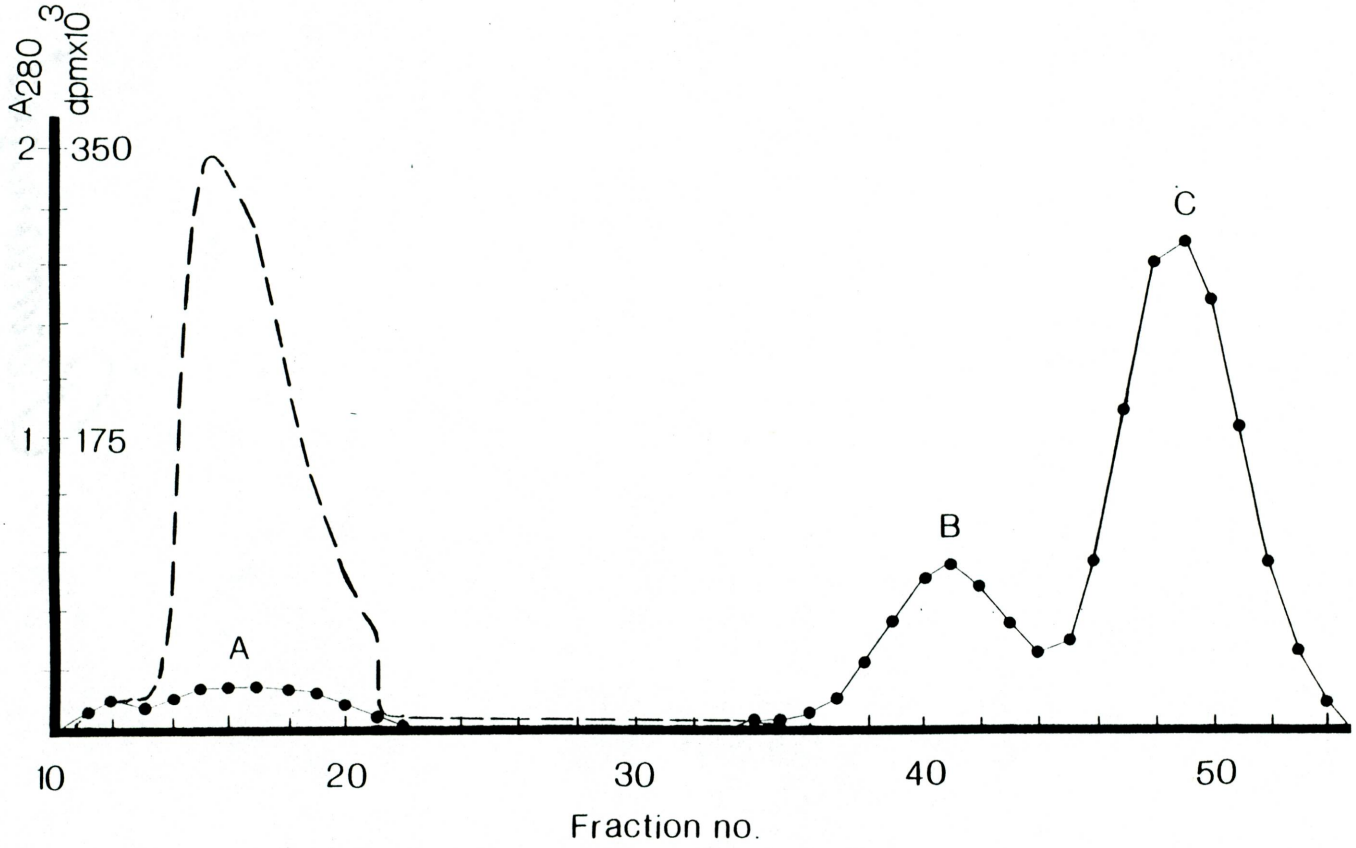

Fig. 11

Figure 12. Column chromatography of human hemoglobin (Type IV) on Sephadex G-75-40 gel after incubated with 0.01 mM ^{3}H-primaquine, mouse liver microsomes, and cofactors *in vitro*. All fractions were counted for radioactivity. -------, A_{280}; ●———●, radioactivity in dpm.

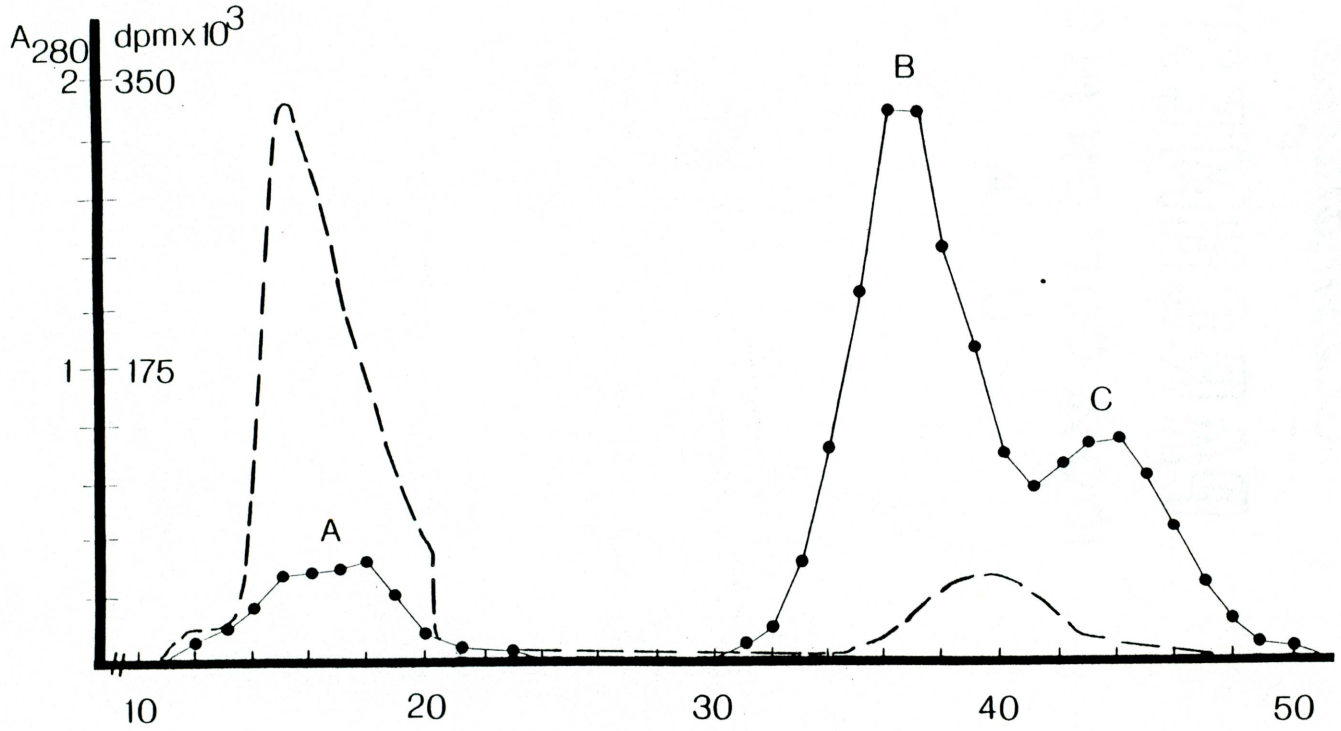

Fig. 12

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